REMARKS/ARGUMENTS

With entry of this amendment, claims 78-81, 83-85, 132 and 135 are pending, claim 82 having been canceled. Claims 79, 80 and 132 stand withdrawn from further consideration.

Claims 78, 81, 83-84 and 135 are amended to correct certain obvious typographical errors and for consistency of usage of certain abbreviations, *e.g.*, β-APP and β-APPsw. Support for the additional amendments to claims 81 and 83 is provided at, *e.g.*, page 54, lines 8-15. No new matter is added by these amendments.

No amendment should be construed as an acquiescence of any rejection.

Objections

Specification

Applicants acknowledge the objection to claims 78 and 84 has been withdrawn.

The Examiner alleges the amended description of Figure 5 is unclear, and proposed alternate wording. Without acquiescing to the object, but to proceed with more compact prosecution of this case, Applicants amend the description of Figure 5 generally in conformance with the Examiner's suggestion. A minor typographical error, the omission of β adjacent to secretase, is also corrected.

Applicants also amend the description of Figure 5 to recite that the polynucleotide region corresponding to amino acids 46-501 of SEQ ID NO:43 is set forth in SEQ ID NO:42, and not SEQ ID NO:44. The description of Figure 5 is also amended to further point out that the active enzyme portion includes an internal peptide region (SEQ ID:56) and a transmembrane region (SEQ ID NO:62). Support for these amendments is provided, *e.g.*, in original Figure 5, at page 10, lines 20-21 and at page 11, lines 10-11 and 17-18. No new matter is added by these amendments.

Applicants request the Examiner reconsider and withdraw the objection to the Specification.

Claims

Claim 82 stands objected to for reciting the phrase "β-amyloid precursor protein (β-APP)," which is allegedly unnecessary. Claims 78, 83 and 84 stand objected to for a lack of consistency in the abbreviation "β-APP." Applicants amend claims 78, 82, 83 and 84 in accordance with the Examiner's suggestion. Applicants submit these amendments do not change the scope of these claims.

Claim 135 stands objected to for using the word "comprised" instead of "comprising." Applicants amend claim 135 in accordance with the Examiner's suggestion. Applicants submit this amendment does not change the scope of this claim.

Applicants therefore request the Examiner reconsider and withdraw the objection to these claims.

Rejections

35 U.S.C. § 101

Applicants acknowledge the rejection of claims 81 and 82 under 35 U.S.C. § 101 has been withdrawn.

35 U.S.C. § 112, Second Paragraph

Applicants acknowledge the rejection of claims 78 and 81-83 under 35 U.S.C. § 112, second paragraph, has been withdrawn.

Claims 78 and 84 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly confusing. The Examiner says the references to SEQ ID NO: 103 and 104 are confusing because those sequences do not identify human beta amyloid precursor protein wild-type and its Swedish mutation, but their fragments that are beta secretase substrates.

Applicants do not understand the basis for this rejection. "The essential inquiry ... is whether the claims set out and circumscribe a particular subject matter with a reasonable degree of clarity and precision. Definiteness of claim language must be analyzed, not in a vacuum, but in light of: (A) The content of the particular application disclosure; (B) The

teachings of the prior art; and (C) The claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made." MPEP § 2173.01.

The Specification describes that MBP-C125 substrates can be the last 125 amino acids of ß-APP fused to the carboxy-terminal end of maltose binding protein (*see* Specification, page 68, lines 15-17). MBP-125wt refers to the substrate having the wild type cleavage site (*see* Specification, page 68, lines 20-22). MBP-125sw refers to the substrate having the Swedish double mutation (*see* Specification, page 68, lines 20-22). SEQ ID NO: 103 and 104 refer to the wild type and Swedish double mutation cleavage sites, respectively. Thus, the SEQ ID NOs: refer to the cleavage sites in the protein. Applicants believe this meaning is clear from the specification (*see* Specification, page 68, lines 15-50 and Figure 19).

Without acquiescing to the objection, but to proceed with more compact prosecution of this case, Applicants amend claims 78 and 84 to delete the SEQ ID NOs. Applicants submit this amendment does not change the scope of these claims.

35 U.S.C. § 112, Written Description

Applicants acknowledge the rejection of claim 82 as allegedly lacking written description is withdrawn.

35 U.S.C. § 112, Enablement

Claims 78, 81-85 and 135 stand rejected under 35 U.S.C. § 112, first paragraph. The Examiner acknowledges the specification is enabling for *in vitro* testing, but alleges the specification does not reasonably provide enablement for *in vivo* testing.

Initially, the Examiner says the claims are directed to a method of *in vitro* and *in vivo* testing a chemical compound for its ability to inhibit beta secretase, wherein the *in vitro* part of the method uses as substrates polypeptides of SEQ ID NO: 104 and 83. Applicants respectfully disagree. Claim 78, as amended, recites using a protein purified to apparent homogeneity comprising a segment of a β-secretase enzyme protein wherein (i) the segment lacks the signal sequence (amino acid residues 1-22 with respect to SEQ ID NO:2) and the

putative pro region (amino acid residues 23-45 with respect to SEQ ID NO:2). The protein has β-secretase activity as evidenced by an ability to cleave a substrate selected from the group consisting of the 695 amino acid isotype of beta amyloid precursor protein (β-APP) between amino acids 596 and 597 thereof, MBP-C125wt and MBP-C125sw. The method is not limited to these particular substrates. Further, the method is not limited to SEQ ID NO:104 and 83 as substrates, but instead any β-secretase substrate having a β-secretase cleavage site can be used.

The office action first repeats the grounds of rejection from the previous office action (pp. 5-7). Applicants responded in the amendment of November 3, 2003, and Applicants' remarks will not be repeated here. The office action then provides the Examiner's response to Applicants' comments (pp. 7-10). The Examiner's response can be subdivided into a number of issues, which will be addressed in turn.

The Examiner responds and says the claims are not enabled for animal subjects and transgenic animal subjects. While Applicants disagree, Applicants amend claim 81 to recite a transgenic mouse. Applicants submit the rejection is moot in view of this amendment.

The Examiner also responds and says the Specification allegedly does not enable any way of administration of any chemical compound that can considered as a candidate inhibitor of β-secretase. Applicants respectfully disagree. Whether a patent claim is supported by the disclosure in an application requires a determination of whether that disclosure, as filed, provides sufficient guidance to a person of ordinary skill in the art to make and use the claimed invention. The specification need not disclose, however, what is well-known in the relevant art. *In re Buchner*, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991). Indeed, the "specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled [in the art] and already available to the public." Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, First Paragraph – Enablement Chemical/Biotechnical Applications, Section III, A., 2, a., ii, (b) (1997). *See also* MPEP § 2164.01 and § 2164.08. Further, there is no requirement to incorporate by reference publications that describe subject matter well known in the art.

Applicants again point to page 54, line 16 to page 55, line 3, of the specification which discusses the administration of test compounds, including the routes of administration, dose ranges, and methods well know in the art to determine routes of administration and dosage ranges. For example, the specification teaches that "routes of administration and dosage ranges can be determined empirically, using methods well know in the art" and refers to a publication by Benet *et al.*, *Pharmacokinetics* in *Good & Gilman's The Pharmacological Basis of Therapeutics*, Ninth Edition, as applied to standard animal models, such as a transgenic PDAPP animal model. *See* Specification, page 54, lines 26-31. Based on this disclosure, and the knowledge of the skilled artisan, the selection of the proper solvent, dose, frequency and time of administration and toxicity profiles can be routinely determined.

The Examiner also says the specification does not define the meaning of the term "acceptable toxicity profile." Applicants respond that the specification need not define terms that are well known in the art. MPEP § 2111.01. For example, Applicants submit abstracts by LoRusso *et al.* ("Phase I clinical trial of pyrazolacridine NSC366140 (PD115934)", *Clin. Cancer Res.* 1487-93 (1995); Ito *et al.* ("Phase II study of paclitaxel (BMS-181339) intraveneously infused over 3 hours for advanced or metastatic breast cancer in Japan. BMS-181339 Breast Cancer Study Group"; *Invest. New Drugs* 16(2):183-90 (1998); and Wong *et al.* ("FK 506 rescue therapy of intractable liver allograft rejection", *Transpl. Int.* 7(Supp1):S70-6 (1994)), all of which confirm the term "acceptable toxicity profile" had an art-accepted meaning at the time of Applicants' filing date. Copies of these abstracts are submitted for the convenience of the Examiner.

Further, the specification need not contain specific examples if the invention is otherwise disclosed in such a manner that one skilled in the art will practice it without an undue amount of experimentation. MPEP § 2164.02. Only objective enablement is required. Applicants submit the skilled artisan, familiar with the testing of compounds in mice, could select the routes of administration, dosage ranges and methods well known in the art to determine such routes of administration and dosage ranges.

The Examiner also responds that the specification is silent as to how to measure cognitive ability and Aβ deposits. Applicants respectfully disagree for the reasons set forth above. Further, Applicants again point to page 54, line 16 to page 55, line 3, of the specification which describes well known and characterized animals models of Alzheimer's disease. As described in the specification, such animal models can be used to detect prophylactic and/or therapeutic efficacy of test compounds by standard assays for disease conditions associated with Alzheimer's disease (*e.g.*, Aβ elevation, amyloid plaque formation and cognitive impairment). Such assays are well known in the art.

For example, Hsaio *et al* ("Correlative Memory Deficits, Aβ Elevation, and Amyloid Plaques in Transgenic Mice," *Science* 274: 99-102 (1996)) describe tests for amyloid deposits and also describe a simple memory test for cognitive impairment, the Morris water maze (*see* page 100). Using these tests, Hsaio *et al.* establish a correlation between memory deficits and amyloid plaque formation. The skilled artisan would appreciate, in view of Applicants' specification, that the Morris water maze and other tests of cognitive ability could be used in the instantly claimed method.

Further, Morgan *et al.* ("A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease," *Nature* 408(6815):982-5 (2000)) and Janus *et al.* ("A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease," *Nature* 408(6815):979-82 (2000)) published after the priority date of the instant application, confirm Applicants' discovery that a reduction in amyloid plaque formation is correlated with improved cognition in a mouse model. Importantly, both Morgan *et al.* and Janus *et al.* use a Morris water maze test to cognitive ability (*see* Morgan *et al.*, page 984; Janus *et al.*, page 981, left column). Applicants submit such references confirm enablement of the claims.

The Examiner also states WO 96/40896 is not incorporated by reference nor listed in the information disclosure statement. For the convenience of the Examiner, Applicants submit a copy of WO 96/40896 and also copies of Hsiao *et al.*, Morgan *et al.*, and Janus *et al.*

Applicants request the Examiner reconsider and withdraw this rejection.

35 U.S.C. § 103

Applicants acknowledge the rejection of claims 78 and 81-85 has been withdrawn.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,

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17. K. Schwarz, G. H. Gauss, Z. Li, S. Desiderio, M. R. Lieber, unpublished results.
18. G. H. Gauss and M. R. Lieber, *Mol. Cell. Biol.* **16**, 258 (1996).
19. Immunophenotypes of patients with *RAG* mutations were obtained from routine clinical specimens at the admission of the patients. B cells (CD20) and T cells (CD3) are expressed as a percentage of total peripheral blood mononuclear cells (PBMCs): P1: 0% CD20, 0% CD3; P2: 0% CD20, 15% CD3; P3: 0% CD20, 59% CD3; P4: 0% CD20, 15% CD3; P5: 0% CD20, 0% CD3; and P6: 1% CD20, 70% CD3. The CD3-positive cells in P2, P4, and P6 were identified as maternal T cells by HLA typing. The CD3-positive cells in P3 were of patient origin as assessed by HLA classification and minisatellite analysis. The CD3 cells were not revertants because no wild-type *RAG* signal was detected in PCR or SSCP in MNCs of the patient. V_α and V_β repertoires were addressed by reverse transcriptase–PCR in PBMCs and exhibited an oligoclonal pattern. Thus, patient P3 was considered leaky.
20. W.-C. Lin and S. Desiderio, *Science* **260**, 953 (1993).
21. Y. Ichikara, M. Hirai, Y. Kurosawa, *Immunol. Lett.* **33**, 272 (1992).
22. RAG-1 and RAG-2 expression vectors used for all recombination assays were identical except for the single mutation introduced; thus, promotor and 5′ and 3′ untranslated region influences on expression are excluded. Cells transfected with RAG expression vectors were boiled in SDS lysis buffer. Equal amounts of total protein (100 μg) were fractionated by 10% SDS–polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose and detected by immunoblotting with affinity-purified antibodies to RAG as described (*20*).
23. K.S., U.P., D.L., and C.R.B. are recipients of grants of the Sonderforschungsbereich 322 from the Deutsche Forschungsgemeinschaft. G.H.G. is supported by a PHS grant awarded to the Stanford University Program in Cancer Biology. M.R.L. is a Leukemia Society of America Scholar, and research in his laboratory is supported by grants from the NIH and a grant from the Council for Tobacco Research. S.D. is supported by a grant from the National Cancer Institute and by the Howard Hughes Medical Institute.

3 June 1996; accepted 20 August 1996

Correlative Memory Deficits, Aβ Elevation, and Amyloid Plaques in Transgenic Mice

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Transgenic mice overexpressing the 695–amino acid isoform of human Alzheimer β-amyloid (Aβ) precursor protein containing a $Lys^{670} \rightarrow Asn$, $Met^{671} \rightarrow Leu$ mutation had normal learning and memory in spatial reference and alternation tasks at 3 months of age but showed impairment by 9 to 10 months of age. A fivefold increase in Aβ(1–40) and a 14-fold increase in Aβ(1–42/43) accompanied the appearance of these behavioral deficits. Numerous Aβ plaques that stained with Congo red dye were present in cortical and limbic structures of mice with elevated amounts of Aβ. The correlative appearance of behavioral, biochemical, and pathological abnormalities reminiscent of Alzheimer's disease in these transgenic mice suggests new opportunities for exploring the pathophysiology and neurobiology of this disease.

Alzheimer's disease (AD), the most common cause of dementia in aged humans, is a disease of unknown etiology. Amyloid plaques are routinely used for diagnosing AD in brain tissue (*1*), even though other histologic changes such as neurofibrillary tangles, synaptic and neuronal loss, and dystrophic neurites are also usually present and sometimes correlate better with dementia (*2*, *3*). The amyloid in senile plaques is composed of Aβ, a 39– to 43–amino acid protein derived from the larger amyloid precursor protein (APP). Small numbers of classic senile plaques develop in the brain with age, but large numbers of senile plaques are found almost exclusively in patients with Alzheimer's type dementia. A diagnosis of AD is made only if both cognitive deterioration and senile plaques are present (*4*). APP isoforms resulting from alternative splicing form a set of polypeptides ranging from 563 to 770 residues in length. The most abundant of these, APP_{695}, is predominantly expressed in neurons (*5*) and lacks a Kunitz-protease inhibitor (KPI) domain present in the APP_{751} and APP_{770} isoforms. Five mutations in APP, all located in or near the Aβ domain, have been identified in families with early-onset AD (*6*–*10*).

Transgenic mice (Swiss Webster × C57B6/DBA2) expressing three isoforms of mutant APP ($Val^{717} \rightarrow Phe$) with an overrepresentation of KPI-containing isoforms showed Alzheimer-type neuropathology, including abundant thioflavin S–positive Aβ deposits, neuritic plaques, synaptic loss, astrocytosis, and microgliosis (*11*), but deficits in memory and learning have not yet been reported. Transgenic mice (JU) expressing human wild-type APP_{751} showed deficits in spatial reference and alternation tasks by 12 months of age (*12*). However, only 4% of aged (≥12 months) transgenic mice exhibited Aβ deposits, and these were rare and diffuse and did not stain with Congo red dye (*13*). Transgenic mice (FVB/N) overexpressing wild-type and variant human or mouse APP_{695} developed a central nervous system disorder that involved most of the corticolimbic regions of the brain (except the somatosensorimotor area) and resembled an accelerated naturally occurring senescent disorder of FVB/N mice (*14*). Parameters that influence the phenotype of transgenic mice expressing APP include host strain, APP primary structure, and extent of APP expression (*14*). We investigated the effects of APP overexpression in C57B6/SJL F_2 mice backcrossed to C57B6 breeders because of their greater longevity compared with FVB/N mice expressing identical transgenes.

Human APP_{695} containing the double mutation $Lys^{670} \rightarrow Asn$, $Met^{671} \rightarrow Leu$ (K670N,M671L; APP_{770} numbering), which was found in a large Swedish family with early-onset AD (*10*), was inserted into a hamster prion protein (PrP) cosmid vector (*15*) in which the PrP open reading frame (ORF) was replaced with the variant APP ORF [see (*14*)]. The resulting mice, Tg(HuAPP695.K670N-M671L)2576, produced 5.56 ± 0.33 units (mean ± SEM; 73-day-old mice) to 5.76 ± 0.74 units (430-day-old mice) of transgenic brain APP expression, where a unit of expression is equivalent to the amount of endogenous mouse APP in nontransgenic (control) littermates (Fig. 1). Transgenic APP expres-

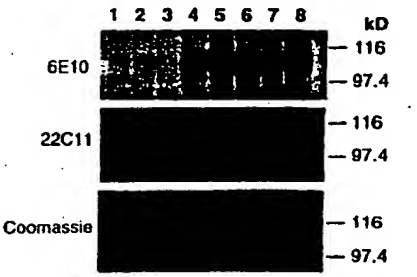

Fig. 1. Brain APP immunoblot of young and old Tg^+ mice and nontransgenic control mice with 6E10 (*24*), which recognizes human but not mouse APP, and 22C11 (Boehringer Mannheim), which recognizes both human and mouse APP. Lanes 1 to 3, nontransgenic mice; lanes 4 to 6, 73-day-old mice; lanes 7 and 8, 430-day-old mice. Detailed methods for APP quantitation were described previously (*14*); antibody binding was revealed with ^{35}S-labeled protein A instead of ^{125}I-labeled protein A.

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sion appeared to remain unchanged between 2 and 14 months of age.

Two groups of 7 to 9 transgene-positive (Tg^+) mice and 10 to 11 transgene-negative (Tg^-) control littermates underwent spatial alternation testing in a Y-maze at 3 and 10 months of age. Three groups of 9 to 13 Tg^+ mice and 10 to 14 Tg^- littermates underwent spatial reference learning and memory testing in the Morris water maze (*16*) at 2, 6, and 9 to 10 months of age. The test experience for each set of mice was novel, and all mice were tested in a coded manner. The 9- to 10-month-old mice were N_1-generation mice (C57B6 × C57B6/SJL F_2); the 2- and 6-month-old mice were N_2-generation mice (C57B6 × C57B6 × C57B6/SJL F_2). A subset of the N_2-generation mice (8 transgenic and 10 control mice) were retested at 12 to 15 months of age.

When transgenic and control mice were given a choice of entering either of two arms in a Y-maze, they tended to alternate their choices spontaneously. Ten-month-old transgenic mice, however, showed significantly less tendency ($P < 0.03$) than did age-matched control mice to alternate the arms on successive choices (Fig. 2F). The behavior of the older transgenic mice on the spatial alternation task was characteristic of animals with damage to the hippocampal formation (*17*).

Nine- to 10-month-old transgenic mice were also impaired in their performance in the water maze relative to age-matched controls (*18*) (Fig. 2). The performance of transgenic mice trained and tested at 2 or 6 months of age was not significantly different from that of age-matched control mice on most measures. The amount of time taken by the mice to reach the hidden platform (the escape latency) did not differ between 2-month-old transgenic and control mice at any point during training, whereas the latency was significantly different on every day for 9- to 10-month-old mice (*19*). Six-month-old transgenic mice differed from controls in escape latency only on the last day of training. After the last training day (day 6), all mice were given a probe trial, in which they swam in the pool for 60 s with the platform removed (*20*). One measure of the animals' knowledge of platform location is the percentage of the 60-s swim spent in the target quadrant (the quadrant that held the platform during training; Fig. 2B). Because the platform is placed in the center of the target quadrant during training, an additional measure that has proven especially useful for mice involves recording the number of times they cross the center of each quadrant. The number of times each mouse crossed the center of the target quadrant (platform crossings; Fig. 2C) and the percentage of total quadrant center crossings that were in the target quadrant were both significantly different [21.5 ± 5.2% for transgenic mice versus 36.1 ± 3.9% for control mice ($P < 0.05$), where 25% is performance at the level of chance] (Fig. 2D) for 9- to 10-month-old transgenic mice compared with age-matched controls.

When 12- to 15-month-old N_2-generation transgenic mice were retested in the water maze (after rearranging the extramaze cues), they showed significantly impaired performance ($P < 0.05$) compared with control littermates on escape latencies after the fifth trial block and on probe trials given after the sixth and ninth trial blocks. These data suggest that the age-related learning impairment seen in N_1-generation Tg^+ mice can occur despite further genetic dilution of the SJL strain. Although the escape latencies of the transgenic N_2-generation mice were significantly longer than those of their control littermates, they were also shorter than those of naïve Tg^+ mice of comparable age. Thus, deficits in escape latency in aged transgenic mice are unlikely to result from difficulty in swimming, as aged mice given sufficient practice can swim as well as younger mice.

Because it is possible that the performance of older transgenic mice was attributable to sensory or motor impairments, we also tested 9- to 10-month-old mice on the visible-platform version of the water maze (Fig. 2E). Although differences in escape latency were evident on the second and fourth of four training days, there were no differences on day 1. These data suggest that although older transgenic mice may show generalized cognitive impairment, they are capable of performing as well as controls when both are relatively naïve. We

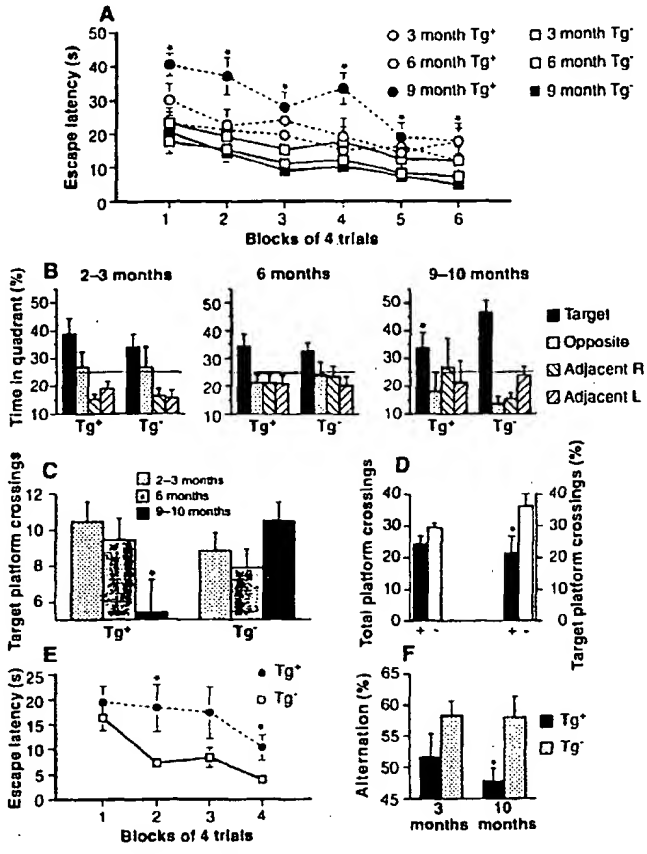

Fig. 2. Learning and memory tests of transgenic and control mice. Asterisks indicate measures in which transgenic mice differed significantly from controls ($P < 0.05$). **(A)** The latency to escape to the hidden platform in the water maze is impaired in Tg^+ mice relative to age-matched nontransgenic controls (*19*). Although the impairment increases with age, Tg^+ mice showed a consistent trend toward longer escape latencies than those of Tg^- controls. **(B)** After 24 trials (over 6 days) with the platform in its fixed location, mice were given a probe trial in which they swam for 60 s with the platform removed. Two- and 6-month-old Tg^- and Tg^+ mice spent significantly more than 25% of their time in the target quadrant, indicating that they had learned its location. Although 9- to 10-month-old control mice still searched selectively for the platform, older transgenic mice spent no more time in the target quadrant than in the other three quadrants, suggesting that they had not learned the platform's location (*20*). **(C)** The implications of (B) are supported by the observation that on probe trials, 9- to 10-month-old Tg^+ mice crossed what had been the exact location of the platform significantly less frequently than did age-matched Tg^- mice. **(D)** The bars on the left indicate that transgenic (+) mice did not differ from control (–) mice in the total number of platform locations crossed (that is, the centers of all four quadrants); the bars on the right show the significant difference between 9- to 10-month-old transgenic mice and controls on the percentage of total platform crossings that were over the target. **(E)** Nine- to 10-month-old Tg^+ mice were also impaired in swimming to a visible platform, although escape latencies did not differ significantly on the first visible-platform training trial. **(F)** Aged Tg^+ mice were impaired in their tendency to spontaneously alternate arm-entry in a Y-maze, another behavioral task sensitive to hippocampal damage.

also compared motor performance of the transgenic and control 9-month-old mice by scoring the total number of times during a probe trial that each mouse crossed imaginary platforms located in each of the four quadrants. If impaired mice swim normally but in a random pattern during probe trials, they should cross the center of all four quadrants combined as many times as would unimpaired mice; they will simply cross the target platform fewer times. If, on the other hand, they are impaired on probe trials simply because they are not swimming, there will be fewer total platform crossings. In fact, the total numbers of platform crossings for transgenic mice (24.4 ± 8.7, mean ± SEM) and control mice (29.5 ± 1.4) were not significantly different, which indicated that motor impairment was not a cause of poor performance in the water maze (Fig. 2D).

After behavioral testing, a subset of each group of mice was killed painlessly. One hemibrain was frozen for cerebral cortical Aβ measurements, and the other hemibrain was immersion-fixed for histopathological analysis. All brains were analyzed in a coded fashion. Measurements of Aβ(1–40) and of Aβ(1–42/43) were done with the use of either the Ban-50/Ba-27 or Ban-50/Bc-05 enzyme-linked immunosorbent assay (ELISA) systems (*21*, *22*). These measurements showed a fivefold increase in the concentration of Aβ(1–40) ($P = 0.03$, rank sum test) and a 14-fold increase in that of Aβ(1–42/43) ($P = 0.03$, rank sum test) between the youngest (2 to 8 months) and oldest (11 to 13 months) Tg^+ mice (Table 1). Thus, there was an association between significantly elevated amounts of Aβ and the appearance of memory and learning deficits in the oldest group of transgenic mice.

Classic senile plaques (with dense amyloid cores) and diffuse deposits were both present in all three mice with elevated Aβ, as determined by ELISA. The Aβ deposits were immunoreactive with antibodies recognizing Aβ(1–5) (*23*), Aβ(1–17) (*24*), Aβ(17–24) (*25*), Aβ(34–40) (*26*), Aβ(42/43) (*27*), and free Aβ42 (*28*). The same plaques were readily identified with multiple antibodies on adjacent sections and were not seen with preimmune or nonspecific ascites, and the immunoreactivity was eliminated by preabsorption with the relevant peptides (Fig. 3). Deposits could not be found in the older or younger controls or in the younger transgenic mice examined. The deposits were found in frontal, temporal, and entorhinal cortex, hippocampus, presubiculum, subiculum, and cerebellum, in a pattern similar to that reported by Games *et al.* (*11*). Dense amyloid plaques were most frequent in cortex, subiculum, and presubiculum. The dense amyloid deposits were readily detected with thioflavin S fluorescence and typically could also be labeled with Congo red to give the characteristic apple-green birefringence of classical amyloid (*29*). Some small deposits had the "Maltese cross" signature pattern of the amyloid cores found in AD brains. Under high magnification, the thioflavin S– and Congo red–positive amyloid plaques usually exhibited wisps or fibers radiating from the central mass, which was often ringed by glial nuclei with both astrocytic and microglial morphology. Glial fibrillary acidic protein–immunoreactive astrocytes were associated with amyloid deposition. Staining by the Gallyas silver method revealed dystrophic neurites surrounding dense core plaques.

In contrast to plaques from patients with sporadic AD, antibodies to β1 and to both free Aβ(42) and Aβ(34–40) (which preferentially recognizes x-40) labeled the ma-

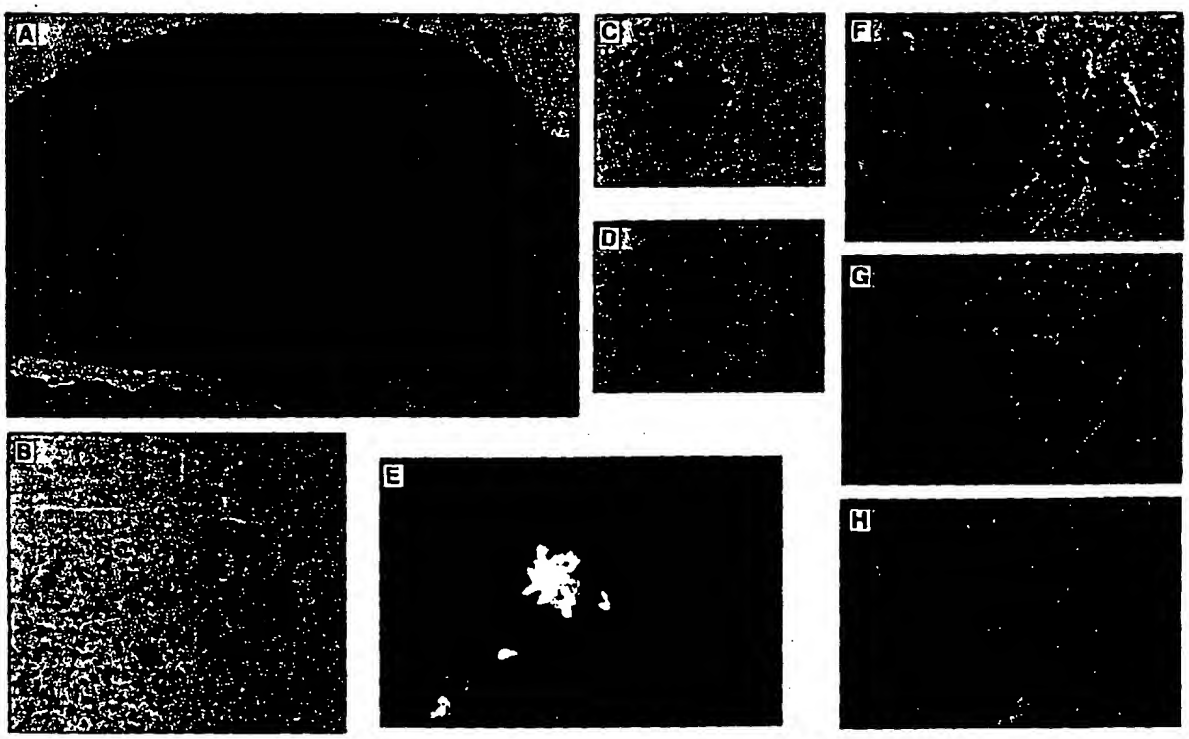

Fig. 3. Extracellular amyloid deposits in transgenic mice A01493 (age, 368 days) and A01488 (354 days) overexpressing human APP_{695} with the K670N,M671L mutation. **(A)** A01493, multiple plaques in the cerebral cortex and subiculum staining with 4G8 mAb. **(B)** A01493, inset from (A). **(C)** A01488, plaque in subiculum staining with 4G8 mAb. **(D)** A01488, plaque in section adjacent to (C) fails to stain with 4G8 mAb preabsorbed with Aβ(14–24). **(E)** A01488, plaques staining with thioflavin S. **(F)** A01488, plaque staining with Aβ(1) affinity-purified antiserum specifically recognizing the NH_2-terminus of Aβ. **(G)** A01488, plaque staining with Aβ(42) affinity-purified antiserum specifically recognizing the COOH-terminus of Aβ(1–42). **(H)** A01488, plaque staining with α40 affinity-purified antiserum specifically recognizing the COOH-terminus of Aβ(1–40). Magnifications: ×100 (A), ×250 (B), ×1000 (C, D, F, and G), ×640 (E), and ×500 (H).

Table 1. *Concentrations of Aβ in transgenic and control mouse brains. Brain tissue was stained with* monoclonal antibody (mAb) 4G8 (*25*), which recognizes both mouse and human Aβ. All amyloid deposits stained with 6E10 (*24*), which specifically recognizes human Aβ. No extracellular 6E10 staining was detected in three 105- to 106-day-old Tg⁺ mice or one 155-day-old Tg⁺ mouse (A01480, A01547, A01548, and Tg2576 founder). ++, 2 to 5 plaques per section; +++, 6 to 10 plaques per section; ++++, >10 plaques per section; –, no staining. Because all the pathological specimens were analyzed in a coded fashion, some nonspecific, equivocal staining that could not be blocked by preabsorption of the antibody with specific peptides was observed in some sections (indicated by ±).

Mouse number	Transgene	Age when killed (days)	Aβ(1–40) (pmol/g)	Aβ(1–42/43) (pmol/g)	Amyloid plaques
		Mice killed at 11 to 13 months of age			
A01484	+	361	325	219	+++
A01488	+	354	192	129	++
A01489	–	354	<2	<2	±
A01492	–	371	<2	<2	–
A01493	+	368	273	177	++++
A01495	–	354	<2	<2	–
A01496	–	354	<2	<2	±
Mean (±SEM) Aβ concentration in Tg⁺ mice:			264 ± 38	175 ± 26	
		Mice killed at 6 to 8 months of age			
A01984	–	233	<2	<2	±
A01987	–	219	<2	<2	–
A01989	+	219	45	18	–
A02561	–	214	<2	<2	
A02595	–	207	<2	<2	
		Mice killed at 2 to 5 months of age			
A02428	–	139	<2	<2	–
A02429	–	139	<2	<2	
A02430	–	139	<2	<2	–
A02565	+	118	71	21	
A02900	–	85	<2	<2	
A03103	+	67	32	2	
A03107	+	67	45	10	
Mean (±SEM) Aβ concentration in Tg⁺ mice:			48 ± 8	13 ± 4	

jority of deposits. This may reflect the $APP_{670-671}$ mutations, which greatly increase cleavage at the β1 site, leading to large concentrations of all fragments beginning with the β1 epitope. In contrast, the Val^{717} → Phe mutations increase the percentage of x-42 (*21*, *30*).

Our results demonstrate the feasibility of creating transgenic mice with robust behavioral and pathological features resembling those found in AD. Impairment in learning and memory became apparent in mice 9 months of age and older; this impairment was correlated with markedly increased amounts of Aβ and was accompanied by numerous amyloid plaques and Aβ deposits. We have demonstrated that an APP transgene lacking the KPI domain is also capable of engendering amyloid plaques in mice. The increase in the concentration of Aβ cannot be explained by a rise in transgenic APP expression, which appeared to remain unchanged with age. Concentrations of Aβ(1–42/43) rose more markedly than did those of Aβ(1–40). This result parallels the finding in humans with presenilin 1 and presenilin 2 mutations showing more significant elevations of Aβ(1–42/43) than of Aβ(1–40) in serum and cultured fibroblasts (*31*). Studies correlating individual performance in learning and memory tests with concentration of Aβ and extent of amyloid deposition may help to ascertain the contribution of each parameter to behavioral deficits. Whether the learning and memory deficits in these mice are caused by or merely correlate with a rise in brain Aβ levels and amyloid deposition remains unresolved.

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18. The water maze was a circular pool (diameter 1 m) filled with water maintained at 29°C and made opaque by the addition of powdered milk. Mice were pretrained by swimming to a 12.7 cm by 12.7 cm Plexiglas platform that was submerged 1.5 cm beneath the surface of the water and placed at random locations within the pool. During pretraining, heavy curtains were drawn around the pool so that mice were unfamiliar with the extramaze room cues on the first day of spatial training. Spatial training consisted of four trials per day, each trial lasting until the mouse reached the platform or 60 s, whichever came first. After each trial, mice remained on the platform for 30 s. Twenty-four hours after the 12th and 24th trials, all mice were subjected to a probe trial in which they swam for 60 s in the pool with the platform removed. Mice were monitored by a camera mounted in the ceiling directly above the pool, and all trials were stored on videotape for subsequent analysis of platform crossings and percent time spent in each quadrant during probe trials. Visible-platform training—in the same pool but with a platform that was black, slightly larger (14.2 cm by 14.2 cm), and raised above the surface of the water—was given at least 24 hours after the second probe trial. The platform location was varied randomly from trial to trial to eliminate the potentially confounding contribution of extramaze spatial cues. In both visible-platform and hidden-platform versions, mice were placed in the pool facing toward the wall of the pool in one of seven randomly selected locations. The numbers of mice tested in the water maze were 12 transgenic and 12 controls at 2 months, 13 transgenic and 14 controls at 6 months, and 9 transgenic and 10 controls at 9 to 10 months of age.
19. The escape latency data were examined with a multifactor analysis of variance (ANOVA) including genotype (transgenic vs. control), age (2 months, 6 months, or 9 to 10 months), and training day (four trials per day). The ANOVA revealed significant main effects of genotype [$F(1, 384) = 65.19$, $P < 0.0001$], age [$F(2, 384) = 7.64$, $P < 0.001$], and trial block [$F(5, 384) = 12.20$, $P < 0.0001$]. Moreover, there was a significant interaction between genotype and age [$F(2, 384) = 10.13$, $P < 0.0001$], indicating that the transgene-induced impairment of escape latency increases with age.
20. All mice were also given a probe trial after 12 training trials (3 days at four trials per day). However, neither the transgenic nor the control mice had learned to search selectively after only 12 trials. The early probe trial was necessary because of the possibility of transient differences manifested only early in training, and because of the likelihood that we would have missed these differences because all behavioral tests were conducted blind to genotype. As none of the mice learned the task, there were no differences among any groups; for the sake of clarity, these data have not been presented graphically.
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Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease

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Much evidence indicates that abnormal processing and extracellular deposition of amyloid-β peptide (Aβ), a proteolytic derivative of the β-amyloid precursor protein (βAPP), is central to the pathogenesis of Alzheimer's disease (reviewed in ref. 1). In the PDAPP transgenic mouse model of Alzheimer's disease, immunization with Aβ causes a marked reduction in burden of the brain amyloid[2,3]. Evidence that Aβ immunization also reduces cognitive dysfunction in murine models of Alzheimer's disease would support the hypothesis that abnormal Aβ processing is essential to the pathogenesis of Alzheimer's disease, and would encourage the development of other strategies directed at the 'amyloid cascade'. Here we show that Aβ immunization reduces both deposition of cerebral fibrillar Aβ and cognitive dysfunction in the TgCRND8 murine model of Alzheimer's disease without, however, altering total levels of Aβ in the brain. This implies that either a ~50% reduction in dense-cored Aβ plaques is sufficient to affect cognition, or that vaccination may modulate the activity/abundance of a small subpopulation of especially toxic Aβ species.

To explore the behavioural consequences of Aβ immunization, we used the TgCRND8 murine model of Alzheimer's disease that expresses a mutant (K670N/M671L and V717F) human βAPP_{695} transgene under the regulation of the Syrian hamster prion promoter on a C3H/B6 strain background (M.A.C. *et al.*, manuscript in preparation). TgCRND8 mice have spatial learning deficits at 3 months of age that are accompanied by both increasing levels of SDS-soluble Aβ and increasing numbers of Aβ-containing amyloid plaques in the brain. Age- and sex-matched TgCRND8 mice and non-Tg littermates in three cohorts were vaccinated at 6, 8, 12, 16 and 20 weeks with either $A\beta_{42}$ or islet-associated polypeptide (IAPP), which has similar biophysical properties to Aβ but is associated with a non-central nervous system (CNS) amyloidosis. Both immunogens were in β-pleated-sheet conformation at the

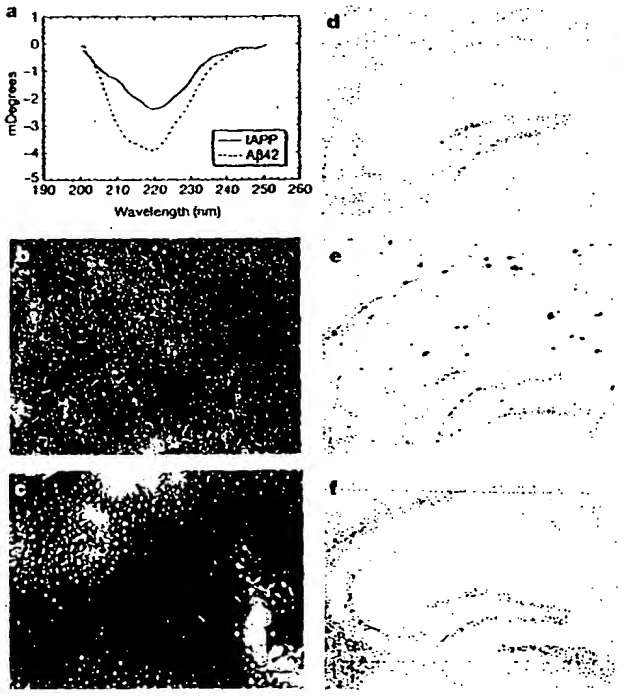

Figure 1 Aβ and IAPP peptide immunogens were predominantly β-structured and induced antibodies recognizing fibrillar Aβ deposits. **a**, Circular dichroism spectra of $A\beta_{42}$ (dotted line) and IAPP (solid line) before immunization are predominantly β-structured. *m*Degrees, millidegrees. **b**, Negative-stain electron microscopy (scale 150 nm) of $A\beta_{42}$ peptide immunogen showed varying length fibres; **c**, IAPP peptide immunogen showed short laterally aggregated fibres. **d**, **f**, Serum (1:1,000 dilution) from non-immunized TgCRND8 mice (**d**) and from 23-week old IAPP-immunized mice (**f**) did not recognize the Aβ plaques in adjacent, non-formic-acid treated sections from a non-immunized TgCRND8 mouse with abundant Aβ-positive plaques. **e**, Sera from $A\beta_{42}$-immunized mice strongly labelled dense-cored amyloid plaques but not diffuse Aβ deposits (which are profusely present in these animals and were labelled with other anti-Aβ antibodies such as 4G8; ref. 28).

time of injection (Fig. 1) and induced detectable antibody titres in all mice by 13 weeks of age (as measured by enzyme-linked immunoabsorbent assay (ELISA) using fibrillar forms of the respective immunogen). These titres increased by a further ~2–3-fold at 23 weeks ($A\beta_{42}$ titres in $A\beta_{42}$-immunized mice (mean ± s.e.m.): 1:3,640 ± 470 at 13 weeks; 1:7,500 ± 1,712 at 23 weeks; IAPP titres in IAPP-immunized mice: 1:3,833 ± 1,167 at 13 weeks; 1:11,500 ± 3661 at 23 weeks). The sera from Aβ-immunized mice intensely decorated extracellular, dense-cored plaque deposits when applied as an immunohistochemical reagent to sections of brain from TgCRND8 mice containing abundant amyloid plaques (which predominantly display Aβ in a β-sheet conformation) or to formic-acid-treated sections (which also display additional non-β-sheet Aβ-epitopes) (Fig. 1). However, these sera reacted only very weakly with diffuse, non-fibrillar Aβ deposits, which can be readily detected in these tissues by anti-Aβ monoclonal antibodies such as 4G8 (data not shown). The Aβ-immune sera did not stain normal neurons, indicating limited crossreactivity with βAPP holoprotein. In contrast, sera from non-immunized or IAPP-immunized mice did not stain any structures. Together, these data indicate that in this strain of mice, immunization with $A\beta_{42}$ protofibrillar assemblies induced antibodies directed primarily towards Aβ in a β-sheet conformation.

The mice were tested longitudinally in a reference memory version of the Morris water maze test at 11, 15, 19 and 23 weeks (Fig. 2). At each age of testing, the hidden platform was placed in a different quadrant of the pool. These data were analysed for the entire test period using a mixed model analysis of variance (ANOVA), with immunogen ($A\beta_{42}$ versus IAPP) and genotype (TgCRND8 versus non-Tg) as a between-subject factor, and age-of-testing (11, 15, 19 and 23 weeks) as a within-subject factor. This longitudinal design and mode of analysis, which simulates longitudinal human clinical trials[4], revealed that $A\beta_{42}$-immunized TgCRND8 mice performed significantly better than IAPP-immunized TgCRND8 mice ($P < 0.05$), with 31% of the performance variance being due to the effects of the immunogen. However, the improvement was partial—the $A\beta_{42}$-immunized TgCRND8 mice did not perform as well as their non-Tg littermates ($P < 0.01$). Because the experimental design involved testing of naive mice at 11 weeks of age, followed by a series of reversal tests at 15, 19 and 23 weeks, an additional analysis carried out on the reversal tests confirmed improved performance for the Aβ-immunized Tg mice ($P < 0.02$) during this phase of testing as well. The overall conclusion that $A\beta_{42}$ immunization ameliorates the cognitive deficit of TgCRND8 mice was robust, regardless of whether the analysis assessed latency to reach the hidden platform or swim path length (a measure that is less sensitive to swim speed and floating[5]).

The improved performance of $A\beta_{42}$-immunized TgCRND8 mice was not due to a nonspecific effect of immunization or to an effect on other behavioural, motor, or perceptual systems. Control studies

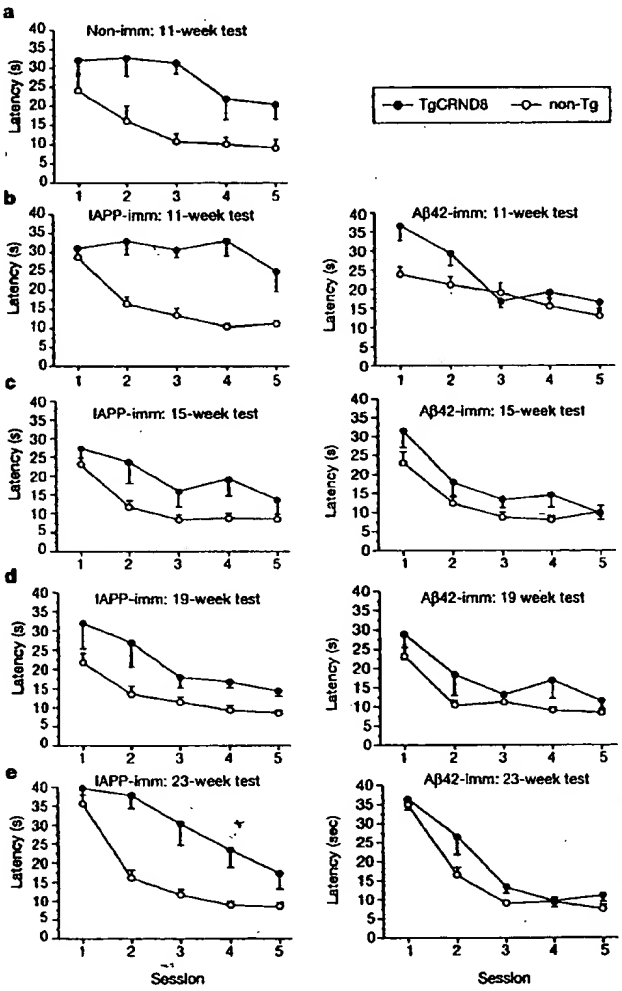

Figure 2 Reference memory version of Morris water maze test in TgCRND8 mice. At 11 weeks of age, non-immunized TgCRND8 mice ($n = 5$) show cognitive impairment relative to non-Tg controls ($n = 8$) (a), which is similar to that of IAPP-immunized TgCRND8 mice ($n = 12$) (b, left), whereas the performance of $A\beta_{42}$-immunized TgCRND8 mice ($n = 9$) (b, right) approaches that of non-Tg littermates ($n = 19$). At 15 (c) and 19 (d) weeks of age, the IAPP-immunized TgCRND8 mice ($n = 6$) (left) were impaired compared with non-Tg littermates ($P < 0.01$, $\omega^2 \approx 36\%$; $n \approx 15$), but were not significantly different from the $A\beta_{42}$-immunized TgCRND8 mice ($n \approx 6$) (right). At 23 weeks of age, the IAPP-immunized TgCRND8 mice ($n = 6$) (e, left) were significantly impaired relative to both non-Tg littermates ($n = 15$; $P < 0.001$, $\omega^2 = 65\%$) and $A\beta_{42}$-immunized TgCRND8 mice ($n = 6$; $P < 0.01$) (e, right). Vertical bars represent s.e.m. See text and Supplementary Information for statistical analyses.

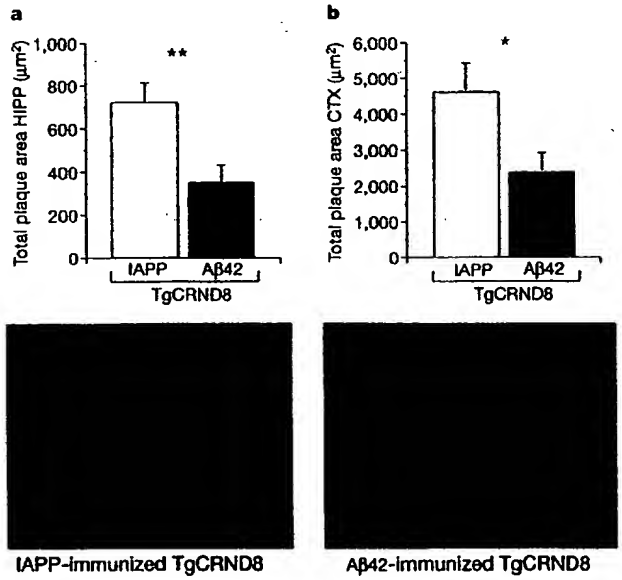

Figure 3 At 25 weeks of age, in TgCRND8 mice immunized with $A\beta_{42}$ peptides, dense-cored Aβ plaque burden is reduced in the hippocampus (HIPP) (a) and in the cerebral cortex (CTX) (b). The $A\beta_{42}$-immunized TgCRND8 mice had 50% fewer plaques than IAPP-immunized TgCRND8 controls (71.4 ± 10.8 per area counted versus 119.7 ± 14.6 in the cortex, $P < 0.05$; and 11.6 ± 1.6 per area counted versus 20.9 ± 1.7 in hippocampus, $P < 0.01$). Representative pictures of the distribution of Aβ plaques labelled by the Dako 6F/3D anti-Aβ monoclonal antibody in hippocampus of IAPP- and $A\beta_{42}$-immunized TgCRND8 mice (c and d, respectively). Vertical bars represent s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$. Scale bars, 100 μm.

in non-Tg mice established that immunization with Freund's Adjuvant plus phosphate buffered saline, Freund's plus $A\beta_{42}$, Freund's plus IAPP, IAPP alone, or $A\beta_{42}$ alone had no effect on performance in the Morris water maze test ($P > 0.1$). During non-spatial pre-training at 11 weeks, the latency of random search for a hidden platform, as well as swim speed, and swim path length to a visible platform were not affected by genotype or immunization ($P > 0.05$). Similarly, at 23 weeks, performance during a visible platform test and spontaneous exploration of the open field were not significantly influenced by immunization ($P > 0.05$). Gender effects were not significant between the TgCRND8 and IAPP- or $A\beta_{42}$-immunized non-Tg littermates ($P > 0.05$).

Analysis of performance in the probe trials with the platform removed (annulus crossing index, passes over platform site or dwelling in the target quadrant) revealed no significant differences owing to immunization or genotype, or interactions between these two factors (each $P > 0.05$). This probably arises both because the probe trials were conducted within 30 min of the final training trial on day 5 (by which point most mice had learned), and because repeated administration of probe trials (at 15, 19 and 23 weeks) makes them a less effective measure of spatial memory[6].

Because the main analysis revealed significant immunogen × genotype ($P < 0.01$) and immunogen × genotype × age ($P < 0.05$) interactions, as well as significant main factor effects for immunogen, genotype and age of testing ($P < 0.01$ for all), *post hoc* analyses were carried out for each age of testing. These showed markedly improved performance in $A\beta_{42}$-immunized TgCRND8 mice at 11 and 23 weeks, with $A\beta_{42}$-immunization accounting for large portions of the variance (ω^2) (19% at 11 weeks; 42% at 23 weeks). Analyses at 15 and 19 weeks did not show statistically significant differentiation of the two immunogens. However, this probably reflects the effects of previous test experience ('carrying-over' effect[7]). Reduction of impairment during re-testing in the water maze has been reported in another study of a murine model of Alzheimer's disease[8], and similar re-test effects are commonly seen in placebo-treated patients in human trials[9]. The subsequent re-emergence of impaired cognition in the IAPP-immunized TgCRND8 mice at 23 weeks probably reflects the advance of Alzheimer's-disease-related phenotypes, which are clearly progressive over this epoch in TgCRND8 mice. Thus, increased Aβ levels are first detectable by western blot or ELISA analysis in brain homogenates at 8.5–17 and 10 weeks, respectively, but thereafter rise progressively. Similarly, cortical dense-cored Aβ-plaques are first detectable immunohistochemically at 7–10 weeks, but increase by a further 18-fold at 19–27 weeks (M.A.C. *et al.*, manuscript in preparation).

In agreement with previous reports[2,3], $A\beta_{42}$ immunization caused ~50% reduction in the number and size of Aβ-positive dense-cored plaques (Fig. 3) without affecting steady-state levels of βAPP holoprotein, amino-terminal secreted fragments (βAPP_S), or carboxy-terminal fragments in the brain (see Supplementary Information). However, in contrast to the previous reports, $A\beta_{42}$ immunization had no significant effect on the levels of formic-acid-extractable Aβ in brain at 13 weeks ($P > 0.1$) or 25 weeks (IAPP immunized ($n = 7$): $A\beta_{40} = 11{,}475 \pm 1{,}567$ fmol per mg protein, $A\beta_{42} = 39{,}983 \pm 4{,}387$; $A\beta_{42}$ immunized ($n = 6$): $A\beta_{40} = 12{,}276 \pm 2{,}386$; and $A\beta_{42} = 50{,}457 \pm 8552$, mean ± s.e.m., $P \approx 0.27$ two-tailed *t*-test). Sandwich ELISAs using two other detection antibodies (directed at Aβ residues 1–4 and 17–24) yielded similar results, arguing against a selective effect of immunization on Aβ species differing in their amino terminus identity or in post-translational modification.

One explanation for our results is that dense-cored cerebral amyloid plaques are the toxic moiety, and the ~50% reduction caused by $A\beta_{42}$ vaccination is sufficient to prevent or reverse the behavioural deficits. Although this cannot be excluded, the density of such plaques correlates poorly with the ante-mortem severity of dementia in most human studies[10]. Furthermore, a dissociation between plaque deposition and cognitive and/or neuronal dysfunction has been described in other TgAPP mice[11,12]. An alternative explanation is that immunization affects Aβ either in a particular conformation (for example, β-sheet forms in protofibrils) or in a restricted compartment. The former is more likely because we used oligomeric assemblies of Aβ in β-sheets ('protofibrils') as an immunogen, and the resultant antisera preferentially recognized β-sheet forms of Aβ. This is significant because monoclonal antibodies raised to Aβ epitopes that initiate fibril aggregation inhibit assembly of synthetic Aβ oligomeric protofibrils *in vitro*[13]. It is possible, therefore, that the antibodies induced in the current strain of mice may bind to β-sheet oligomeric aggregates and inhibit further assembly. This Aβ species is especially neurotoxic, a critical intermediary in fibrillogenesis and an accurate predictor of neurodegeneration[14–17]. Consequently, small amounts of such antibodies that cross the blood–brain barrier (0.1% of serum levels[3]) might be sufficient to attenuate both the behavioural deficits caused by this neurotoxic form of Aβ and the further aggregation of these species into fibrillar Aβ in dense-cored plaques. Because this pool of Aβ is small, and because antibodies to this form of Aβ might need only inhibit assembly of Aβ fibrils to have a functional effect, these antibodies need not necessarily cause large changes in total cerebral Aβ. It is also possible that the antibodies redistribute Aβ from dense-cored plaques to diffuse Aβ deposits. Either might explain the divergent effects on levels of dense-cored plaques and Aβ measured biochemically (although high levels of cerebral Aβ in the presence of relatively few plaques are seen in humans[18] and in transgenic mouse models[19]).

It is conceivable, however, that immunization might modulate Aβ metabolism through several distinct mechanisms, including destruction of Aβ by microglial phagocytosis[3]. Such different effects, which might reflect variations in antigen presentation or in strain-specific immune response, might also explain the disparity between the effects of Aβ immunization on total Aβ levels in this and the previous studies. If correct, such variations could complicate the use of active immunization in humans. In addition to clinical caveats raised previously[20], it is also important to emphasize that, although $A\beta_{42}$ immunization had a strong effect on behaviour and neuropathology in this mouse model, it did not fully reverse these features. This might reflect either inefficient ingress of antibodies to the CNS and/or the possibility that other βAPP proteolytic fragments may be involved in the pathogenesis of Alzheimer's disease[21]. These issues will need to be addressed directly by future studies. Nonetheless, our data support the hypotheses that Aβ plays a central role in Alzheimer's disease and that procedures that modulate its production, assembly and/or removal might be used as treatments. □

Methods

Mice

The TgCRND8 mice (M.A.C. *et al.*, manuscript in preparation) were maintained in a hybrid C3H/B6 background. Experimental groups derived from crosses to B6 mice were matched for gender and weight, and transgene identity was unknown to experimenters at all stages of the study. Sixty-eight 6-week-old mice were immunized (TgCRND8, $n = 28$, non-Tg, $n = 40$); 60 entered the behavioural testing at 11 weeks (Aβ immunized: TgCRND8, $n = 12$; non-Tg, $n = 20$; and IAPP immunized: TgCRND8, $n = 9$; non-Tg, $n = 19$). Thirteen mice were killed at 13 weeks, and their brains analysed (Aβ immunized: TgCRND8, $n = 3$; non-Tg, $n = 4$; IAPP immunized: TgCRND8, $n = 2$, non-Tg = 4). All remaining mice were longitudinally tested until 23 weeks of age (Aβ immunized: TgCRND8, $n = 9$; non-Tg, $n = 16$; IAPP immunized: TgCRND8, $n = 7$; non-Tg, $n = 15$). Thirteen non-immunized mice (TgCRND8, $n = 5$; non-Tg, $n = 8$) were included as controls in the test conducted at 11 weeks of age.

Immunization procedures

Synthetic $A\beta_{42}$ and IAPP peptides were isolated by reverse phase high performance liquid chromatography on a C_{18} μbondapak column, with purity determined using mass spectrometry and amino-acid analyses. Before preparation of the vaccine, the secondary structure and fibre morphology of the peptides were assessed using circular dichroism spectroscopy and by electron microscopy[22]. The immunization protocol and schedule have been described[2]. Antibody titres were assayed in triplicate by ELISA (see

Supplementary Information for more details) in serum samples (200 µl of blood) collected at 13 and 25 weeks.

Behavioural tests and data analysis

The water maze apparatus, mouse handling and general testing procedures have been described[22]. Before the first spatial learning test at 11 weeks, all mice underwent non-spatial pre-training (NSP) to assess swimming abilities and to accustom mice to the test[24,25] (see Supplementary Information). Two days after the NSP phase, all mice underwent a reference memory training with a hidden platform placed in the centre of one quadrant of the pool for 5 days, with four trials per day. After the last trial of day 5, the platform was removed from the pool and each mouse received one 60-s swim probe trial. Escape latency (s), length of swim path (cm), swim speed (cm s^{-1}), % of floating (speed less then 5 cm s^{-1}), % of time in outer zone (near the pool wall), and % of time and path in each quadrant of the pool were recorded using an on-line HVS image video tracking system[23] (see Supplementary Information).

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Analysis of βAPP and amyloid burden in brain

Three 5-µm sections at 25-µm intervals from one cerebral hemisphere were immunostained with Dako 6F/3D anti-Aβ monoclonal antibody to residues 8–17 (which is primarily reactive against dense-cored plaques) with 4G8 (ref. 28), or with sera from immunized mice, and counterstained with haematoxylin and resin mounted as described (M.A.C. *et al.*, manuscript in preparation). For some samples the formic-acid treatment step was omitted. End products were visualized with diaminobenzidine. Amyloid plaque burden was assessed using Leco *IA-3001* image analysis software interfaced with a Leica microscope and a Hitachi KP-M1U CCD video camera. The quantitative analysis was performed at of ×25 magnification, and the image frame and guard size was set to 0,0,639,479 (307,200 μm^2) for each slide. The brain area (cortex or hippocampus) was outlined using the edit plane function, and the area and number of plaques in the outlined structure were recorded. Data were pooled for all three sections.

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significant difference (LSD), $P < 0.02$), were nearly flawless by trial 5, and performed significantly better than the KLH-vaccinated transgenic mice on both trials 4 and 5 (multiple analysis of variance, MANOVA: $F_{(2,15)} = 5.83$, $P < 0.02$ and $F_{(2,15)} = 12.16$, $P < 0.001$, respectively; KLH transgenic group different from both other groups by Fischer's LSD post hoc comparisons, $P < 0.05$ on trial 4 and $P < 0.01$ on trial 5). Our individual evaluation of the performance of the two transgenic genotypes made it clear that both APP-only and APP+PS1 transgenic mice benefited from the Aβ vaccinations (Fig. 1c).

Serological analysis indicated that mice injected with Aβ developed antibodies against the Aβ peptide. Very high titres were found in both transgenic and nontransgenic mice immunized with Aβ (IC_{50} = 27,000 ± 5,000 and 48,000 ± 18,000, respectively; not significant). There was no anti-Aβ activity in the KLH-immunized transgenic mice, untreated transgenic mice, nor nontransgenic mice at final dilutions of serum down to 1:16, indicating that transgenic mice did not spontaneously generate an antibody reaction to Aβ.

Immunization with Aβ caused a modest reduction in Aβ deposits in the frontal cortex, with a significant reduction in the Congo-red-stained area of APP+PS1 mice, and a significant reduction in the Aβ-immunostained area of APP mice (Fig. 2 and Fig. 3). Reductions of a similar extent were found in hippocampus. We also quantified immunostaining using Aβ40- and Aβ42-specific antisera, both of which exhibited the same modest reductions found in total Aβ immunostaining. We suspect that, with a larger sample size, statistically significant partial reductions would be found in all these measures consistent with other recent reports[9-11]. In general, the percentage reduction in Aβ deposition was greater in the APP mice than the APP+PS1 mice. The absolute reductions were greater, however, in the doubly transgenic animals. The APP+PS1 mice already had substantial Aβ deposits by the time vaccinations were initiated[12]. Further studies will test whether beginning vaccinations at an earlier age, or combining vaccination with other Aβ-lowering treatments, will result in more complete protection from Aβ deposition, and improve the cognitive performance of 15-months-old transgenic mice even further.

Our most important finding here is that Aβ vaccination protects transgenic mice from developing memory deficits compared with KLH-immunized (control) transgenic mice. But how important is the learning paradigm in discerning these differences. We have found that in using the reference-memory version of the water maze, mice of this age (15.5 months) have deficits in escape latency, but not retention on the probe trial[3]. Thus, the more demanding working-memory version of the water-maze task may be essential to detect such differences. Similarly, a spatial task would require intact function of hippocampal and, to a lesser extent, cortical structures, the locations where plaques accumulate earliest and to the greatest extent in these mice[12-14].

This vaccination-associated protection from memory impairment occurs in the presence of reduced, but still substantial Aβ deposits. The mechanism by which immunization with Aβ blocks learning and memory deficits is not understood. One possibility is that the antibodies neutralize Aβ in some restricted compartment or deplete a non-deposited form of Aβ (for example, a soluble form) that is responsible for the memory loss observed. Recently, soluble Aβ has been proposed as the cause of synapse loss in APP transgenic mice, as some transgenic lines develop reductions in synaptophysin immunoreactivity in dentate gyrus without developing Aβ deposits[15]. A second possibility is that microglia activated by the inoculations[1] can clear the deposited Aβ, thereby permitting normal cognitive function. This is not easily reconciled with the relatively modest Aβ clearance detected, although exhaustive regional analyses have yet to be completed. Perhaps even mice that have already developed extensive brain pathology and memory deficits can benefit from vaccinations given later in life. In view of the absence of adverse effects on behaviour and brain functioning, and the protection of memory functions by the Aβ vaccines, we strongly recommend testing of this and related approaches for the treatment and prevention of Alzheimer's disease. □

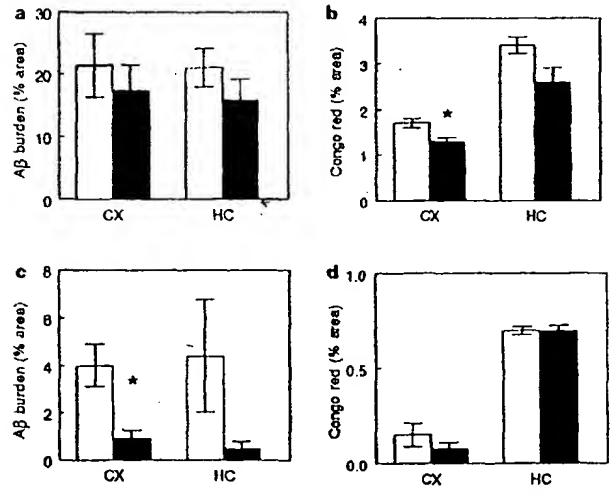

Figure 3 Measurement of amyloid histopathology after Aβ peptide immunization. **a**, **b**, Results for the APP+PS1 mice; **c**, **d**, results for APP-only transgenic mice. A significant reduction in Congo-red staining in frontal cortex was found in APP+PS1 mice vaccinated with Aβ ($n = 4$) compared to in APP+PS1 mice vaccinated with KLH ($n = 5$; **b**). There was a significant reduction in Aβ immunostaining in APP-only transgenic mice vaccinated with Aβ ($n = 3$) compared to in KLH-vaccinated APP mice ($n = 2$; **c**). *, $P < 0.05$; **, $P < 0.01$ by *t*-test. CX, frontal cortex; HC, hippocampus.

Methods

Vaccination protocols

Mice were obtained by breeding Tg 2576 APP transgenic mice[16] with PS1 line 5.1 transgenic mice[17], resulting in nontransgenic, APP, APP+PS1 and PS1 transgenic mice as described by us previously[12,13]. Human Aβ1–42 peptide (Bachem) was suspended in pyrogen-free Type I water at 2.2 mg ml^{-1} then mixed with 10 × PBS to yield 1 × PBS and incubated overnight at 37 °C. Control mice were injected with KLH that was prepared in the same manner. The antigen suspension was mixed 1:1 with Freund's complete adjuvant and 100 μg Aβ injected subcutaneously by an experimenter who had no role in the behavioural testing. A boost of the same material (prepared freshly) was made in incomplete Freund's at two weeks and injected once monthly for the next three months. Subsequent monthly boosts were made in mineral oil. Mice were vaccinated, beginning at 7.5 months of age. The sample size of each group was: 6 (3 female/3 male) nontransgenic mice vaccinated with Aβ or KLH; 7 (4 female/3 male) transgenic mice vaccinated with Aβ; 7 (4 female/3 male) transgenic mice vaccinated with KLH. The first post-vaccination behavioural testing period was started 5 days after the fifth vaccination at 11.5 months of age. The second behavioural testing period was started at 15.5 months of age, one month after the ninth vaccination. Mice were killed at 16 months of age. We note that transgenic and nontransgenic mice were also tested for performance in the radial-arm water maze at 6 months of age (before vaccination) and all mice performed well.

Radial-arm water maze testing

Experimenters were unaware of the experimental conditions of the mice at the time of testing. The maze consisted of a circular pool 1 m in diameter with six swim alleys (arms) 19 cm wide that radiated out from an open central area (40 cm in diameter), with a submerged escape platform located at the end of one of the arms[3,18]. Spatial cues were present on the walls and ceiling of the testing room. The escape platform was placed in a different arm each day, forcing mice to use working memory to solve the task. Each day, mice were given the opportunity to learn the location of the submerged platform during four consecutive acquisition trials followed 30 min later by a retention trial (trial 5). On each trial, the mouse was started in one arm not containing the platform and allowed to swim for up to one minute to find the escape platform. Upon entering (all four paws within the swim alley) an incorrect arm or failing to select an arm after 20 s, the mouse was gently pulled back to the start arm for that trial and charged an error. All mice spent 30 s on the platform following each trial before beginning the next trial. On subsequent trials that day, the start arm was varied, so the mouse could not simply learn the motor rule 'second arm to the left', but must learn the spatial location of the platform that day. After the fourth trial was completed, the mice were placed in their home cage for 30 min, then returned to the maze and administered the retention trial. The platform was located in the same arm on each trial within a day, and was in a different arm across days. Over 1–2 weeks of

training, control groups gradually improved performance as they learned the procedural aspects of the task, reaching an asymptotic level of 0.5–1 errors on trials 4 and 5. In the experiments presented here mice were trained until the nontransgenic mice reached asymptotic performance: 9 days at 11.5 months or 11 days at 15.5 months. The scores for each mouse on the last two days of testing were averaged and used for statistical analysis. Sensorimotor tests identified no differences among these groups in open field behaviour or string agility testing. As in earlier work, all transgenic mice were impaired on the balance beam, a deficit observed as early as six months of age[5], but this deficit was not modified by Aβ vaccination.

ELISA analysis for serum antibodies

Ninety-six-well Immulon 4HBX (Dynex) micro plates were coated with the Aβ1–42 protein (250 ng per well) for 1 h at 37 °C. They were washed four times with 0.45% NaCl + 0.05% Tween-20 (washing buffer, WB). The plates were blocked with 5% non-fat dry milk (NFDM) in PBS overnight at 4 °C and washed the following day. Mouse serum was prepared in PBS at an initial dilution of 1:16 and subsequent twofold dilutions were made. All samples were run in duplicate and incubated at 37 °C for 1 h followed by washing 10 times in WB. Plates were blocked a second time with 5% NFDM in PBS for 30 min at 37 °C followed by washing five times before the addition of an anti-mouse IgG HRP-conjugate. The secondary antibody was diluted 1:5,000 in PBS and incubated for 1 h at 37 °C. Plates were then washed 10 times in WB and developed with 3,3,5,5-tetramethylbenzidine substrate (Sigma) in perborate buffer (Sigma). The reaction was stopped with 2 M sulphuric acid. Plates were read spectrophotometrically at 450 nm. The anti-Aβ1–42 antibody titre was defined as the reciprocal of the dilution of antisera that produced 50% of the maximum signal detected for that sample.

Histopathology

Mice were overdosed with pentobarbital, perfused with saline and their brains removed. One hemisphere was immersion-fixed in fresh, buffered paraformaldehyde for 24 h. Frozen sections were stained for Aβ peptides by immunohistochemistry[13,19] or for Congo red. The area of frontal cortex occupied by stain was measured with a Videometric V150 image analysis system (Oncor) on a Nikon Microphot FX microscope. Stained regions were measured using HSI segmentation by an experimenter unaware of the subject condition. Both stain intensity and area were measured, although only areas are reported here as this is the convention for Aβ deposits ('amyloid burden'). The results were not qualitatively different when evaluating area, stain intensity or their product (total immunoreactivity[19]). Data were collected from equally spaced horizontal sections for both frontal cortex (anterior to the corpus callosum; 12 per mouse) and hippocampus (10 per mouse).

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Induction of vanilloid receptor channel activity by protein kinase C

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Capsaicin or vanilloid receptors (VRs) participate in the sensation of thermal and inflammatory pain[1–3]. The cloned (VR1) and native VRs are non-selective cation channels directly activated by harmful heat, extracellular protons and vanilloid compounds[4–8]. However, considerable attention has been focused on identifying other signalling pathways in VR activation; it is known that VR1 is also expressed in non-sensory tissue[1,9] and may mediate inflammatory rather than acute thermal pain[3]. Here we show that activation of protein kinase C (PKC) induces VR1 channel activity at room temperature in the absence of any other agonist. We also observed this effect in native VRs from sensory neurons, and phorbol esters induced a vanilloid-sensitive Ca^{2+} rise in these cells. Moreover, the pro-inflammatory peptide, bradykinin, and the putative endogenous ligand, anandamide, respectively induced and enhanced VR activity, in a PKC-dependent manner. These results suggest that PKC may link a range of stimuli to the activation of VRs.

PKC is a prominent participant in pain signalling. Targeted deletion of PKC-ϵ in mice[10] markedly attenuates thermal- and acid-induced hyperalgesia. In turn, activation of PKC-ϵ potentiates heat-evoked currents in sensory neurons[11,12]. Further, the algesic peptide, bradykinin, potentiates heat responses[11,12], induces depolarization[13–16], and evokes secretion[17–19] from vanilloid-sensitive neurons in a PKC-dependent manner. However, the molecular targets for these effects have not yet been clearly identified. We therefore investigated whether these actions of PKC are mediated by VRs. Rat VR1 was expressed in *Xenopus laevis* oocytes and studied using a two-electrode voltage clamp technique. Treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) to activate endogenous PKC increased the amplitude of currents evoked by capsaicin (Fig. 1a, c), anandamide (Fig. 1b, c) and protons (extracellular pH 5; data not shown). In addition, TPA by itself produced a slowly developing current (Fig. 1a, b) that was not observed in uninjected oocytes ($n = 5$) or oocytes expressing the NMDA (*N*-methyl D-aspartate) receptor ($n = 8$). These actions were probably mediated by PKC because no responses were elicited by the inactive TPA analogue, 4α-phorbol ($n = 4$), and responses to TPA were inhibited by the selective PKC inhibitor[20], bisindolylmaleimide (BIM, 200 nM, Fig. 1c).

Next, we examined whether the current induced by TPA alone was mediated by VR1. In these experiments VR1-expressing oocytes were treated separately with either TPA or capsaicin, to avoid cross-sensitization. Figure 1d shows the response of a TPA-treated oocyte to a series of depolarizing pulses from −80 mV to +80 mV. Outwardly rectified currents were evoked that were similar to those

1: Invest New Drugs. 1998;16(2):183-90.

Phase II study of paclitaxel (BMS-181339) intravenously infused over 3 hours for advanced or metastatic breast cancer in Japan. BMS-181339 Breast Cancer Study Group.

Ito Y, Horikoshi N, Watanabe T, Sasaki Y, Tominaga T, Okawa T, Tabei T, Kuraishi Y, Tamura K, Abe R, Kitajima M, Yamaguchi S, Kobayashi T, Koyama H, Orita K, Takashima S, Nomura Y, Ogawa M.

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A Phase II study of paclitaxel in patients with primary advanced or metastatic breast cancer was conducted by a cooperative study group consisting of 16 institutions in Japan. Paclitaxel at a dose of 210 mg/m2 was intravenously infused over 3 hours, along with premedication to prevent hypersensitivity reactions. The course was repeated at 21-day intervals. Of 62 eligible patients, 60 were evaluable for toxicity and 59 were evaluable for efficacy. Forty-five patients were previously treated with anthracyclines. Twenty-one of 59 patients (35.6%) had a major objective response including 2 CRs and 19 PRs (95% confidence interval, 23.6-49.1%). A response rate of 35.5% (CR1, PR10) was observed in 31 patients refractory to the anthracyclines containing prior metastatic chemotherapy. Median (range) time was 41 (6-100) days to onset of and median duration of response was 125 (36-305) days. Toxicities included leukopenia (grade 3, 4: 67%), anemia (grade 1-3: 80%), thrombocytopenia (grade 1: 8%), alopecia (grade 3: 43%), peripheral neuropathy (grade 1-3: 93%), arthralgia (59%), myalgia (46%), nausea and vomiting (40%), fever (33%), allergic reaction (grade 3: 2%) and hypotension (grade 3: 5%). All toxicities were tolerable and manageable. Paclitaxel intravenously infused over 3 hours demonstrated a significant antitumor activity for metastatic breast cancer. Furthermore, paclitaxel exhibited non-cross resistance to anthracycline. Paclitaxel administered as a convenient 3-hour infusion is effective for patients with metastatic breast cancer and has an acceptable toxicity profile.

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1: Clin Cancer Res. 1995 Dec;1(12):1487-93.

Phase I clinical trial of pyrazoloacridine NSC366140 (PD115934).

LoRusso P, Foster BJ, Poplin E, McCormick J, Kraut M, Flaherty L, Heilbrun LK, Valdivieso M, Baker L.

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The pyrazoloacridine (PZA) analogue NSC366140 (PD115934) entered clinical trial based on unique preclinical characteristics including solid tumor selectivity in vitro, marked antitumor activity in vivo against murine solid tumors, selectivity against noncycling cells, and activity against multidrug-resistant tumor cells. After identification of the pre-clinical efficacy and an acceptable toxicity profile, a Phase I study of PZA was carried out. A total of 28 patients was entered and received a total of 67 treatment courses. The drug was administered via a 1-h infusion every 21 days. The starting dose was 30 mg/m2 with 2-fold dose escalations through 480 mg/m2. The next dose escalation was 50%, to 720 mg/m2. Grade I through grade IV toxicities were observed. Since no dose-limiting toxicities were observed at 480 mg/m2, and up to grade IV toxicities were observed at 720 mg/m2, an intermediate dose, 600 mg/m2, was evaluated. Dose-limiting toxicities at 720 mg/m2 were hematological (grade III and IV neutropenia) in four of six patients and neurological (up to grade III cerebral toxicities, including restlessness, dizziness, agitation/anxiety, personality changes, and nightmares, as well as myoclonus) in three of six patients treated. The pharmacokinetic parameters which helped predict these toxicities included area under the curve and peak plasma level. Pharmacokinetic studies showed interpatient variations in all parameters studied. The mean area under the curve levels of PZA at the highest two dose levels in patients were near the level detected in mice at their maximum tolerated total dose. The recommended starting dose for Phase II trials using this schedule is 600 mg/m2.

Publication Types:

- Clinical Trial
- Clinical Trial, Phase I

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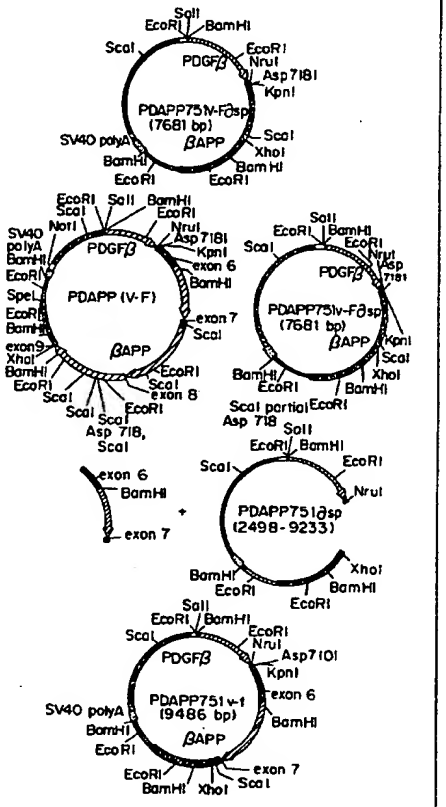
(54) Title: METHOD FOR IDENTIFYING ALZHEIMER'S DISEASE THERAPEUTICS USING TRANSGENIC ANIMAL MODELS

(57) Abstract

The construction of transgenic animal models of human Alzheimer's disease, and methods of using the models to screen potential Alzheimer's disease therapeutics, are described. The models are characterized by pathologies similar to pathologies observed in Alzheimer's disease, based on expression of all three forms of the β-amyloid precursor protein (APP), APP695, APP751, and APP770, as well as various point mutations based on naturally occurring mutations, such as the London and Indiana familial Alzheimer's diseae (FAD) mutations at amino acid 717, predicted mutations in the APP gene, and truncated forms of APP that contain the Aβ region. Animal cells can be isolated from the transgenic animals or prepared using the same constructs with standard techniques such as lipofection or eletroporation. The transgenic animals, or animal cells, are used to screen for compounds altering the pathological course of Alzheimer's disease as measured by their effect on the amount of APP, β-amyloid peptide, and numerous other Alzeimer's disease markers in the animals, the neuropathology of the animals, as well as by behavioral alterations in the animals.



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METHOD FOR IDENTIFYING ALZHEIMER'S DISEASE THERAPEUTICS USING TRANSGENIC ANIMAL MODELS

Background of the Invention

Transgenic animal models of Alzheimer's disease are described along with a method of using the transgenic animal models to screen for therapeutics useful for the treatment of Alzheimer's disease.

Alzheimer's disease (AD) is a degenerative disorder of the brain first described by Alios Alzheimer in 1907 after examining one of his patients who suffered drastic reduction in cognitive abilities and had generalized dementia (*The early story of Alzheimer's Disease*, edited by Bick *et al.* (Raven Press, New York 1987)). It is the leading cause of dementia in elderly persons. AD patients have increased problems with memory loss and intellectual functions which progress to the point where they cannot function as normal individuals. With the loss of intellectual skills the patients exhibit personality changes, socially inappropriate actions and schizophrenia (*A Guide to the Understanding of Alzheimer's Disease and Related Disorders*, edited by Jorm (New York University Press, New York 1987). AD is devastating for both victims and their families, for there is no effective palliative or preventive treatment for the inevitable neurodegeneration.

The impact of AD on society and on the national economy is enormous. It is expected that the demented elderly population in the United States will increase by 41% by the year 2000. It is expensive for the health care systems that must provide institutional and ancillary care for the AD patients at an estimated annual cost of $40 billion (Jorm (1987); Fisher, "Alzheimer's Disease", New York Times, August 23, 1989, page D1, edited by Reisberg (The Free Press, New York & London 1983)). These factors imply action must be taken to generate effective treatments for AD.

At a macroscopic level, the brains of AD patients are usually smaller, sometimes weighing less than 1,000 grams. At a microscopic level, the histopathological hallmarks of AD include neurofibrillary tangles (NFT), neuritic plaques, and degeneration of neurons. AD patients exhibit degeneration of nerve cells in the frontal and temporal cortex of the cerebral

cortex, pyramidal neurons of hippocampus, neurons in the medial, medial central, and cortical nuclei of the amygdala, noradrenergic neurons in the locus coeruleus, and the neurons in the basal forebrain cholinergic system. Loss of neurons in the cholinergic system leads to a consistent deficit in cholinergic presynaptic markers in AD (Fisher (1983); *Alzheimer's Disease and Related Disorders, Research and Development* edited by Kelly (Charles C. Thomas, Springfield, IL. 1984)). In fact, AD is defined by the neuropathology of the brain.

AD is associated with neuritic plaques measuring up to 200 μm in diameter in the cortex, hippocampus, subiculum, hippocampal gyrus, and amygdala. One of the principal constituents of neuritic plaques is amyloid, which is stained by Congo Red (Fisher (1983); Kelly (1984)). Amyloid plaques stained by Congo Red are extracellular, pink or rust-colored in bright field, and birefringent in polarized light. The plaques are composed of polypeptide fibrils and are often present around blood vessels, reducing blood supply to various neurons in the brain.

Various factors such as genetic predisposition, infectious agents, toxins, metals, and head trauma have all been suggested as possible mechanisms of AD neuropathy. However, available evidence strongly indicates that there are distinct types of genetic predispositions for AD. First, molecular analysis has provided evidence for mutations in the amyloid precursor protein (APP) gene in certain AD-stricken families (Goate *et al. Nature* 349:704-706 (1991); Murrell *et al. Science* 254:97-99 (1991); Chartier-Harlin *et al. Nature* 353:844-846 (1991); Mullan *et al., Nature Genet.* 1:345-347 (1992)). Additional genes for dominant forms of early onset AD reside on chromosome 14 and chromosome 1 (Rogaev *et al., Nature* 376:775-778 (1995); Levy-Lahad *et al., Science* 269:973-977 (1995); Sherrington *et al., Nature* 375:754-760 (1995)). Another loci associated with AD resides on chromosome 19 and encodes a variant form of apolipoprotein E (Corder, *Science* 261:921-923 (1993).

Amyloid plaques are abundantly present in AD patients and in Down's Syndrome individuals surviving to the age of 40. The overexpression of APP

in Down's Syndrome is recognized as a possible cause of the development of AD in Down's patients over thirty years of age (Rumble *et al.*, *New England J. Med.* 320:1446-1452 (1989); Mann *et al.*, *Neurobiol. Aging* 10:397-399 (1989)). The plaques are also present in the normal aging brain, although at a lower number. These plaques are made up primarily of the amyloid β peptide (Aβ; sometimes also referred to in the literature as β-amyloid peptide or β peptide) (Glenner and Wong, *Biochem. Biophys. Res. Comm.* 120:885-890 (1984)), which is also the primary protein constituent in cerebrovascular amyloid deposits. The amyloid is a filamentous material that is arranged in beta-pleated sheets. Aβ is a hydrophobic peptide comprising up to 43 amino acids. The determination of its amino acid sequence led to the cloning of the APP cDNA (Kang *et al.*, *Nature* 325:733-735 (1987); Goldgaber *et al.*, *Science* 235:877-880 (1987); Robakis *et al.*, *Proc. Natl. Acad. Sci.* 84:4190-4194 (1987); Tanzi *et al.*, *Nature* 331:528-530 (1988)) and genomic APP DNA (Lemaire *et al.*, *Nucl. Acids Res.* 17:517-522 (1989); Yoshikai *et al.*, *Gene* 87, 257-263 (1990)). A number of forms of APP cDNA have been identified, including the three most abundant forms, APP695, APP751, and APP770. These forms arise from a single precursor RNA by alternate splicing. The gene spans more than 175 kb with 18 exons (Yoshikai *et al.* (1990)). APP contains an extracellular domain, a transmembrane region and a cytoplasmic domain. Aβ consists of up to 28 amino acids just outside the hydrophobic transmembrane domain and up to 15 residues of this transmembrane domain. Thus, Aβ is a cleavage product derived from APP which is normally found in brain and other tissues such as heart, kidney and spleen. However, Aβ deposits are usually found in abundance only in the brain.

The larger alternate forms of APP (APP751, APP770) consist of APP695 plus one or two additional domains. APP751 consists of all 695 amino acids of APP695 plus an additional 56 amino acids which has homology to the Kunitz family of serine protease inhibitors (KPI) (Tanzi *et al.* (1988); Weidemann *et al.*, *Cell* 57:115-126 (1989); Kitaguchi *et al.*, *Nature* 331:530-532 (1988); Tanzi *et al.*, *Nature* 329:156 (1987)). APP770

contains all 751 amino acids of APP751 and an additional 19 amino acid domain homologous to the neuron cell surface antigen OX-2 (Weidemann *et al.* (1989); Kitaguchi *et al.* (1988)). Unless otherwise noted, the amino acid positions referred to herein are the positions as they appear in APP770. The amino acid number of equivalent positions in APP695 and APP751 differ in some cases due to the absence of the OX-2 and KPI domains. By convention, the amino acid positions of all forms of APP are referenced by the equivalent positions in the APP770 form. Unless otherwise noted, this convention is followed herein. Unless otherwise noted, all forms of APP and fragments of APP, including all forms of Aβ, referred to herein are based on the human APP amino acid sequence. APP is post-translationally modified by the removal of the leader sequence and by the addition of sulfate and sugar groups.

Van Broeckhaven *et al.*, *Science* 248:1120-1122 (1990), have demonstrated that the APP gene is tightly linked to hereditary cerebral hemorrhage with amyloidosis (HCHWA-D) in two Dutch families. This was confirmed by the finding of a point mutation in the APP coding region in two Dutch patients (Levy *et al.*, *Science* 248:1124-1128 (1990)). The mutation substituted a glutamine for glutamic acid at position 22 of the Aβ (position 618 of APP695, or position 693 of APP770). In addition, certain families are genetically predisposed to Alzheimer's disease, a condition referred to as familial Alzheimer's disease (FAD), through mutations resulting in an amino acid replacement at position 717 of the full length protein (Goate *et al.* (1991); Murrell *et al.* (1991); Chartier-Harlin *et al.* (1991)). These mutations co-segregate with the disease within the families and are absent in families with late-onset AD. This mutation at amino acid 717 increases the production of the $A\beta_{1-42}$ form of Aβ from APP (Suzuki *et al.*, *Science* 264:1336-1340 (1994)). Another mutant form contains a change in amino acids at positions 670 and 671 of the full length protein (Mullan *et al.* (1992)). This mutation to amino acids 670 and 671 increases the production of total Aβ from APP (Citron *et al.*, *Nature* 360:622-674 (1992)).

There are no robust animal models to study AD, although aging nonhuman primates seem to develop amyloid plaques of Aβ in brain parenchyma and in the walls of some meningeal and cortical vessels. Although aged primates and canines can serve as animal models, they are expensive to maintain, need lengthy study periods, and are quite variable in the extent of pathology that develops.

There are no spontaneous animal mutations with sufficient similarities to AD to be useful as experimental models. Various models have been proposed in which some AD-like symptoms may be induced by electrolysis, transplantation of AD brain samples, aluminum chloride, kainic acid or choline analogs (Kisner *et al.*, *Neurobiol. Aging* 7:287-292 (1986); Mistry *et al.*, *J Med Chem* 29:337-343 (1986)). Flood *et al.*, *Proc. Natl. Acad. Sci.* 88:3363-3366 (1986), reported amnestic effects in mice of four synthetic peptides homologous to the Aβ. Because none of these share with AD either common symptoms, biochemistry or pathogenesis, they are not likely to yield much useful information on etiology or treatment.

Several transgenic rodent lines have been produced that express either the human APP gene or human APP complementary DNA regulated by a variety of promoters. Transgenic mice with the human APP promoter linked to *E. coli* β-galactosidase (Wirak *et al.*, *The EMBO J* 10:289-296 (1991)) as well as transgenic mice expressing the human APP751 cDNA (Quon *et al.*, *Nature* 352:239-241 (1991)) or subfragments of the cDNA including the Aβ (Wirak *et al.*, *Science* 253:323-325 (1991); Sandhu *et al.*, *J. Biol. Chem.* 266:21331-21334 (1991); Kawabata *et al.*, *Nature* 354:476-478 (1991)) have been produced. Results obtained in the different studies appear to depend upon the source of promoter and the protein coding sequence used. For example, Wirak *et al.*, *Science* 253:323-325 (1991), found that in transgenic mice expressing a form of the Aβ, intracellular accumulation of "amyloid-like" material, reactive with antibodies prepared against Aβ were observed but did not find other histopathological disease symptoms. The intracellular nature of the antibody-reactive material and the lack of other symptoms suggest that this particular transgenic animal is not a faithful model system

for Alzheimer's disease. Later studies have shown that similar staining is seen in non-transgenic control mice and Wirak *et al.*, *Science* 253:323-325 (1991) was partially retracted in a comment in *Science* 255:143-145 (1992). Thus, the staining seen by Wirak *et al.* appears to be artifactual.

Kawabata *et al.* (1991) report the production of amyloid plaques, neurofibrillary tangles, and neuronal cell death in their transgenic animals. In each of these studies, Aβ or a fragment containing Aβ was expressed. Wirak *et al.* (1991), used the human APP promoter while Kawabata *et al.* (1991) used the human thy-1 promoter. However, Kawabata *et al.* (1991) was later retracted by Kawabata *et al.*, *Nature* 356:23 (1992) and Kawabata *et al.*, *Nature* 356:265 (1992). In transgenic mice expressing the APP751 cDNA from the neuron-specific enolase promoter of Quon *et al.* (1991), rare, small extracellular deposits of material reactive with antibody prepared against synthetic Aβ were observed. A review of the papers describing these early transgenic mice indicate that do not produce characteristic Alzheimer pathologies (see Marx, *Science* 255:1200-1202 (1992)).

Transgenic mice expressing APP751 from a neuron-specific enolase (NSE) promoter were recently described by McConlogue *et al.*, *Neurobiol. Aging* 15:S12 (1994), Higgins *et al.*, *Ann Neurol.* 35:598-607 (1995), Mucke *et al.*, *Brain Res.* 666:151-167 (1994), Higgins *et al.*, *Proc. Natl. Acad. Sci. USA* 92:4402-4406 (1995), and U.S. Patent 5,387,742 to Cordell. Higgins *et al.*, *Ann Neurol.* 35:598-607 (1995) describe results with the same mice as described by Quon *et al.* (1991). Such mice have only sparse Aβ deposits which are more typical of very early AD and young Down's syndrome cases. The deposits seen in this transgenic mouse were also seen, although at a lower abundance, in non-transgenic control animals. Mature lesions such as frequent compacted plaques, neuritic dystrophy and extensive gliosis are not seen in these mice (Higgins *et al.*, *Ann Neurol.* 35:598-607 (1995)). McConlogue *et al.* (1994) reported finding no Aβ deposits in these mice.

Transgenic mice in which APP is expressed from the neuronal specific synaptophysin promoter express APP at low levels equivalent to that in brain

tissue from the NSE APP mice described above. These mice were also reported not to display any brain lesions (Higgins *et al.*).

Transgenic mice containing yeast artificial chromosome (YAC) APP constructs have also been made (Pearson and Choi, *Proc. Natl. Acad. Sci. USA* 90:10578-10582 (1993); Lamb *et al.*, *Nature Genetics* 5:22-30 (1993); Buxbaum *et al.*, *Biochem. Biophys. Res. Comm.* 197:639-645 (1993)). These mice contain the entire human APP genomic gene and express human APP protein at levels similar to endogenous APP; higher levels of expression than that obtained in mice using the NSE promoter. None of these mice, however, show evidence of pathology similar to AD.

Alzheimer's disease animal models, including transgenic models, have been recently reviewed by Lannfelt *et al.*, *Behavioural Brain Res.* 57:207-213 (1993), and Fukuchi *et al.*, *Ann. N.Y. Acad. Sci.* 695:217-223 (1993). Lannfelt *et al.* points out that none of the prior transgenic animals that show apparent plaques demonstrate neuropathological changes characteristic of AD. Lannfelt *et al.* also discusses possible reasons for the "failure" of previous transgenic animal models. Similarly, Fukuchi *et al.* discusses the failure of prior transgenic animal models to display most of the characteristics known to be associated with AD. For example, the transgenic mouse reported by Quon *et al.* is reported to produce Aβ immunoreactive deposits that stain only infrequently with thioflavin S and not at all with Congo Red, in contrast to the staining pattern of AD Aβ deposits.

Alzheimer's disease is characterized by numerous changes in the expression levels of various proteins, the biochemical activity and histopathology of brain tissue, as well as cognitive changes in affected individuals. Such characteristic changes associated with AD have been well documented. The most prominent change, as noted above, is the deposition of Aβ into amyloid plaques (Haass and Selkoe, *Cell* 75:1039-1042 (1993)). A variety of other molecules are also present in plaques, such as apolipoprotein E, laminin, amyloid P component, and collagen type IV (Kalaria and Perry, *Brain Research* 631:151-155 (1993); Ueda *et al.*, *Proc. Natl. Aca. Sci. USA* 90:11282-11286 (1993)). Changes in cytoskeletal

markers have also been associated with AD, such as the changes in microtubule-associated protein tau, MAP-2 or neurofilaments (Kosik *et al.*, *Science* 256: 780-783 (1992); Lovestone and Anderton, *Current Opinion in Neurology & Neurosurgery* 5:883-888 (1992); Brandan and Inestrosa, *General Pharmacology* 24:1063-1068 (1993); Trojanowski *et al.*, *Brain Pathology* 3:45-54 (1993); Masliah *et al.*, *American Journal of Pathology* 142:871-882 (1993)). Alzheimer's disease is also known to stimulate an immunoinflammatory response, increasing such inflammatory markers as glial fibrillary acidic protein (GFAP), α2-macroglobulin, and interleukins 1 and 6 (IL-1 and IL-6) (Frederickson and Brunden, *Alzheimer Disease and Associated Disorders* 8:159-165 (1994); McGeer *et al.*, *Canadian Journal of Neurological Sciences* 18:376-379 (1991); Wood *et al.*, *Brain Research* 629:245-252 (1993)). Finally, neuronal and neurotransmitter changes have been associated with AD, such as the cholinergic, muscarinic, serotinergic, adrenergic, and adensosine receptor systems (Rylett *et al.*, *Brain Res* 289:169-175 (1983); Sims *et al.*, *Lancet* 1:333-336 (1980); Nitsch *et al.*, *Science* 258:304-307 (1992); Masliah and Terry, *Clinical Neuroscience* 1:192-198 (1993); Greenamyre and Maragos, *Cerebrovascular and Brain Metabolism Reviews* 5:61-94 (1993); McDonald and Nemeroff, *Psychiatric Clinics of North America* 14:421-422 (1991); Mohr *et al.*, *Journal of Psychiatry & Neuroscience* 19:17-23 (1994)).

It is therefore an object of the present invention to provide an animal model for Alzheimer's disease that is constructed using transgenic technology.

It is a further object of the present invention to provide transgenic animals characterized by certain genetic abnormalities in the expression of the amyloid precursor protein.

It is a further object of the present invention to provide transgenic animals exhibiting one or more histopathologies similar to those of Alzheimer's disease.

It is a further object of the present invention to provide transgenic animals expressing one or more Aβ-containing proteins at high levels in brain tissue.

It is a further object of the present invention to provide a method of screening potential drugs for the treatment of Alzheimer's disease using transgenic animal models.

Summary of the Invention

The construction of transgenic animal models for testing potential treatments for Alzheimer's disease is described. The models are characterized by a greater similarity to the conditions existing in naturally occurring Alzheimer's disease, based on the ability to control expression of one or more of the three major forms of the β-amyloid precursor protein (APP), APP695, APP751, and APP770, or subfragments thereof, as well as various point mutations based on naturally occurring mutations, such as the FAD mutations at amino acid 717, and predicted mutations in the APP gene. The APP gene constructs are prepared using the naturally occurring APP promoter of human, mouse, or rat origin, efficient promoters such as human platelet derived growth factor β chain (PDGF-B) gene promoter, as well as inducible promoters such as the mouse metallothionine promoter, which can be regulated by addition of heavy metals such as zinc to the animal's water or diet. Neuron-specific expression of constructs can be achieved by using the rat neuron specific enolase promoter.

The constructs are introduced into animal embryos using standard techniques such as microinjection or embryonic stem cells. Cell culture based models can also be prepared by two methods. Cells can be isolated from the transgenic animals or prepared from established cell cultures using the same constructs with standard cell transfection techniques.

The constructs disclosed herein generally encode all or a contiguous portion of one of the three forms of APP: APP695, APP751, or APP770, preferably an Aβ-containing protein, as described herein. Examples of Aβ-containing proteins are proteins that include all or a contiguous portion of APP770, APP770 bearing a mutation in amino acid 669, 670, 671, 690, 692,

and/or 717, APP751, APP751 bearing a mutation in amino acid 669, 670, 671, 690, 692, and/or 717, APP695, and APP695 bearing a mutation in amino acid 669, 670, 671, 690, 692, and/or 717, where each of these Aβ-containing proteins includes amino acids 672 to 714 of human APP. Some specific constructs that are described employ the following protein coding sequences: the APP770 cDNA; the APP770 cDNA bearing a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; the APP751 cDNA containing the KPI protease inhibitor domain without the OX-2 domain in the construct; the APP751 cDNA bearing a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; the APP695 cDNA; the APP695 cDNA bearing a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; APP695, APP751, or APP770 cDNA truncated at amino acid 671 or 685, the sites of β-secretase or α-secretase cleavage, respectfully; APP cDNA truncated to encode amino acids 646 to 770 of APP; APP cDNA truncated to encode amino acids 646 to 770 of APP and including at least one intron; the APP leader sequence followed by the Aβ region (amino acids 672 to 714 of APP) plus the remaining carboxy terminal 56 amino acids of APP; the APP leader sequence followed by the Aβ region plus the remaining carboxy terminal 56 amino acids with the addition of a mutation at amino acid 717; the APP leader sequence followed by the Aβ region; the Aβ region plus the remaining carboxy terminal 56 amino acids of APP; the Aβ region plus the remaining carboxy terminal 56 amino acids of APP with the addition of a mutation at amino acid 717; a combination cDNA/genomic APP gene construct; a combination cDNA/genomic APP gene construct with the addition of a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a combination cDNA/genomic APP gene construct truncated at amino acid 671 or 685; and an APP cDNA construct containing at least amino acids 672 to 722 of APP.

These protein coding sequences are operably linked to leader sequences specifying the transport and secretion of the encoded Aβ related protein. A preferred leader sequence is the APP leader sequence. These

combined protein coding sequences are in turn operably linked to a promoter that causes high expression of Aβ in transgenic animal brain tissue. A preferred promoter is the human platelet derived growth factor β chain (PDGF-B) gene promoter. Additional constructs include a human yeast artificial chromosome construct controlled by the PDGF-B promoter; a human yeast artificial chromosome construct controlled by the PDGF-B promoter with the addition of a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; the endogenous mouse or rat APP gene modified through the process of homologous recombination between the APP gene in a mouse or rat embryonic stem (ES) cell and a vector carrying the human APP cDNA bearing a mutation at amino acid position 669, 670, 671, 690, 692, 717, or a combination of these mutations, such that sequences in the resident rodent chromosomal APP gene beyond the recombination point (the preferred site for recombination is within APP exon 9) are replaced by the analogous human sequences bearing a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations. These constructs can be introduced into the transgenic animals and then combined by mating of animals expressing the different constructs.

The transgenic animals, or animal cells, are used to screen for compounds altering the pathological course of Alzheimer's disease as measured by their effect on the amount and/or histopathology of Alzheimer's disease markers in the animals, as well as by behavioral alterations. These markers include APP and APP cleavage products; Aβ; other plaque related molecules such as apolipoprotein E, laminin, and collagen type IV; cytoskeletal markers, such as spectrin, tau, neurofilaments, and MAP-2; inflammatory markers, such as GFAP, α2-macroglobulin, IL-1, and IL-6; and neuronal and synaptic neurotransmitter related markers, such as GAP43 and synaptophysin, and those associated with the cholinergic, muscarinic, serotinergic, adrenergic, and adensosine receptor systems.

Brief Description of the Drawings

The boxed portions of the drawings indicate the amino acid coding portions of the constructs. Filled portions indicate the various domains of the

protein as indicated in the Figure Legend. Lines indicate sequences in the clones that are 5' or 3' untranslated sequences, flanking genomic sequences, or introns. The break in the line to the left of the constructs in Figures 7 and 8 indicates the presence of a long DNA sequence.

Figure 1a is a schematic of the APP770 cDNA coding sequence.

Figure 1b is a schematic of the APP770 cDNA coding sequence bearing a mutation at position 717.

Figure 2a is a schematic of the APP751 cDNA coding sequence.

Figure 2b is a schematic of the APP751 cDNA coding sequence bearing a mutation at position 717.

Figure 3a is a schematic of the APP695 coding sequence.

Figure 3b is a schematic of the APP695 cDNA coding sequence bearing a mutation at position 717.

Figure 4a is a schematic of a coding sequence for the carboxy terminal portion of APP.

Figure 4b is a schematic of a coding sequence for the carboxy terminal portion of APP bearing a mutation at position 717.

Figure 5 is a schematic of a coding sequence for the Aβ portion of APP.

Figure 6a is a schematic of a combination cDNA/genomic coding sequence allowing alternative splicing of the KPI and OX-2 exons.

Figure 6b is a schematic of a combination cDNA/genomic coding sequence bearing a mutation at position 717 and allowing alternative splicing of the KPI and OX-2 exons.

Figure 7a is a schematic of a human APP YAC coding sequence.

Figure 7b is a schematic of a human APP YAC coding sequence bearing a mutation at position 717.

Figures 8a and 8b are schematics of genetic alteration of the mouse APP gene by homologous recombination between the mouse APP gene in a mouse ES cell and a vector carrying the human APP cDNA (either of the wild-type (Figure 8a) or FAD mutant form (Figure 8b)) directed to the exon 9 portion of the gene. As a result of this recombination event, sequences in

the resident mouse chromosomal APP gene beyond the recombination point in exon 9 are replaced by the analogous human sequences.

Figure 9 is a schematic map of the PDAPP vector, a combination cDNA/genomic APP construct.

Figure 10 is a diagram of the genomic region of APP present in the PDAPP construct. The sizes of original introns 6, 7 and 8, as well as the sizes of the final introns are indicated on the diagram. The locations of the deletions in introns 6 and 8 present in the PDAPP construct are also indicated.

Figure 11 is a diagram of the intermediate constructs used to construct the APP splicing cassette and the PDAPP vector.

Figure 12 is a diagram of the PDAPP-wt vector and the plasmids used to make the PDAPP-wt vector.

Figure 13 is a diagram of the PDAPP-Sw/Ha vector and the plasmids and intermediate constructs used to make the PDAPP-Sw/Ha vector.

Figure 14 is a diagram of the $PDAPP695_{V-F}$ vector and the plasmids and intermediate constructs used to make the $PDAPP695_{V-F}$ vector.

Figure 15 is a diagram of the $PDAPP751_{V-F}$ vector and the plasmids and intermediate constructs used to make the $PDAPP751_{V-F}$ vector.

Detailed Description of the Invention

The constructs and transgenic animals and animal cells are prepared using the methods and materials described below.

Sources of materials.

Restriction endonucleases are obtained from conventional commercial sources such as New England Biolabs (Beverly, MA.), Promega Biological Research Products (Madison, WI.), and Stratagene (La Jolla CA.). Radioactive materials are obtained from conventional commercial sources such as Dupont/NEN or Amersham. Custom-designed oligonucleotides for site-directed mutagenesis are available from any of several commercial providers of such materials such as Bio-Synthesis Inc., Lewisville, TX. Kits for carrying out site-directed mutagenesis are available from commercial suppliers such as Promega Biological Research Products and Stratagene.

Clones of cDNA including the APP695, APP751, and APP770 forms of APP mRNA were obtained directly from Dr. Dmitry Goldgaber, NIH. Libraries of DNA are available from commercial providers such as Stratagene, La Jolla, CA., or Clontech, Palo Alto, CA. PC12 and 3T3 cells were obtained from ATCC (#CRL1721 and #CCL92, respectively). An additional PC12 cell line was obtained from Dr. Charles Marotta of Harvard Medical School, Massachusetts General Hospital, and McLean Hospital. Standard cell culture media appropriate to the cell line are obtained from conventional commercial sources such as Gibco/BRL. Murine stem cells, strain D3, were obtained from Dr. Rolf Kemler (Doetschman *et al.*, *J. Embryol. Exp. Morphol.* 87:27 (1985)). Lipofectin for DNA transfection and the drug G418 for selection of stable transformants are available from Gibco/BRL.

Definition of APP cDNA clones.

The cDNA clone APP695 is of the form of cDNA described by Kang *et al.*, *Nature* 325:733-735 (1987), and represents the most predominant form of APP in the brain. The cDNA clone APP751 is of the form described by Ponte *et al.*, *Nature* 331:525-527 (1988). This form contains an insert of 168 nucleotides relative to the APP695 cDNA. The 168 nucleotide insert encodes the KPI domain. The cDNA clone APP770 is of the form described by Kitaguchi *et al. Nature* 331:530-532 (1988). This form contains an insert of 225 nucleotides relative to the APP695 cDNA. This insert includes the 168 nucleotides present in the insert of the APP751 cDNA, as well as an addition 57 nucleotide region that does not appear in APP751 cDNA. The 225 nucleotide insert encodes for the KPI domain as well as the OX-2 domain. All three forms arise from the same precursor RNA transcript by alternative splicing. The 168 nucleotide insert is present in both APP751 cDNA and APP770 cDNA.

The sequence encoding APP695 is shown in SEQ ID NO:1. This sequence begins with the first base of the initiation codon AUG and encodes a 695 amino acid protein. The region from nucleotide 1789 to 1917 of SEQ ID NO:1 encodes the Aβ. The amino acid sequence of APP695 is shown in SEQ ID NO:2. Amino acids 597 to 639 of SEQ ID NO:2 form the Aβ. The

amino-acid composition of the APP695 is A57, C12, D47, E85, F17, G31, H25, I23, K38, L52, M21, N28, P31, Q33, R33, S30, T45, V62, W8, Y17 resulting in a calculated molecular weight of 78,644.45. These sequences are derived from Kang *et al.* (1988).

The sequence encoding APP751 is shown in SEQ ID NO:3. This sequence begins with the first base of the initiation codon AUG and encodes a 751 amino acid protein. Nucleotides 866 to 1033 of SEQ ID NO:3 do not appear in APP695 cDNA. The region from nucleotide 1957 to 2085 of SEQ ID NO:3 encodes the Aβ. The amino acid sequence of APP751 is shown in SEQ ID NO:4. Amino acids 289 to 345 of SEQ ID NO:4 do not appear in APP695. This 57 amino acid region includes the KPI domain. Amino acids 653 to 695 of SEQ ID NO:4 form the Aβ. These sequences are derived from Ponte *et al.* (1988).

The sequence encoding APP770 is shown in SEQ ID NO:5. This sequence begins with the first base of the initiation codon AUG and encodes a 770 amino acid protein. Nucleotides 866 to 1090 of SEQ ID NO:5 do not appear in APP695 cDNA. Nucleotides 1034 to 1090 of SEQ ID NO:5 do not appear in APP751 cDNA. The region from nucleotide 2014 to 2142 encodes the Aβ. The amino acid sequence of APP770 is shown in SEQ ID NO:6. Amino acids 289 to 364 of SEQ ID NO:6 do not appear in APP695. This 76 amino acid region includes the KPI and OX-2 domains. Amino acids 345 to 364 of SEQ ID NO:6 do not appear in APP751. This 20 amino acid region includes the OX-2 domain. Amino acids 672 to 714 form the Aβ. A probable membrane-spanning region of the APP occurs from amino acid 700 to 723. Unless otherwise stated, all references herein to nucleotide positions refer to the numbering of SEQ ID NO:5. This is the numbering derived from the APP770 cDNA. Unless otherwise stated, all references herein to amino acid positions refer to the numbering of SEQ ID NO:6. This is the numbering derived from APP770. According to this numbering convention, for example, amino acid position 717 refers to amino acid 717 of APP770, amino acid 698 of APP751, and amino acid 642 of APP695. The above sequences are derived from Kang *et al.* (1988) and Kitaguchi *et al.* (1988).

Unless otherwise noted, all forms of APP and fragments of APP, including all forms of Aβ, referred to herein are based on the human APP amino acid sequence. For example, Aβ refers to the human Aβ, APP refers to human APP, and APP770 refers to human APP770. As used herein, the term cDNA refers not only to DNA molecules actually prepared by reverse transcription of mRNA, but also any DNA molecule encoding a protein where the coding region is not interrupted, that is, a DNA molecule having a continuous open reading frame encoding a protein. As such, the term cDNA as used herein provides a convenient means of referring to a protein encoding DNA molecule where the protein encoding region is not interrupted by intron sequences (or any other sequences not encoding protein).

Definition of the APP genomic locus.

Characterization of phage and cosmid clones of human genomic DNA clones listed in Table 1 below originally established a minimum size of at least 100 kb for the Alzheimer's gene. There are a total of 18 exons in the APP gene (Lemaire *et al.*, *Nucl. Acid Res* 17:517-522 (1989); Yoshikai *et al.* (1990); Yoshikai *et al.*, *Nucleic Acids Res* 102:291-292 (1991)). Yoshikai *et al.* (1990) describes the sequences of the exon-intron boundaries of the APP gene. These results taken together indicate that the minimum size of the Alzheimer's gene is 175 kb.

Table 1. Alzheimer's Cosmid and Lambda Clones.

Library	Name of Clone	Insert Size (kb)	Assigned APP Region
Cosmid	1 GPAPP47A	35	25 kb promoter & 9 kb intron 1
	2 GPAAP36A	35	12 kb promoter & 22 kb intron 1
	3 GAPP30A	30-35	5' coding region
	4 GAPP43A	30-35	exons 9, 10 and 11
Lambda	1 GAPP6A	12	exon 6
	2 GAPP6B	18	exons 4 and 5
	3 GAPP20A	20	exon 6
	4 GAPP20B	17	exons 4 and 5
	5 GAPP28A	18	exons 4 and 5
	6 GAPP3A	14	exon 6
	7 GAPP4A	19	exon 6
	8 GAPP10A	16	exons 9, 10 and 11
	9 GAPP16A	21	exon 6

Table 2 indicates where the 17 introns interrupt the APP coding sequence. The numbering refers to the nucleotide positions of APP770 cDNA as shown in SEQ ID NO:5. The starting nucleotide of exon 1 represents the first transcribed nucleotide. It is negative because the +1 nucleotide is the first nucleotide of the AUG initiator codon by convention (Kang *et al.* (1988)). The ending nucleotide of exon 18 represents the last nucleotide present in the mRNA prior to the poly(A) tail (Yoshikai *et al.* (1990)). It has been discovered that Yoshikai *et al.* (1990) and Yoshikai *et al.* (1991) contain an error in the location of exon 8. Figure 1 of Yoshikai *et al.* (1991) includes an EcoRI fragment between EcoRI fragments containing exon 7 and exon 8. In fact, this intervening EcoRI fragment is actually located immediately after exon 8, so that the EcoRI fragment containing exon 7 and the EcoRI fragment containing exon 8 are adjacent to each other.

Table 2. Location of Introns in APP Gene Sequence.

	Starting nucleotide	Ending nucleotide	Following Intron
Exon 1	-146	57	Intron 1
Exon 2	58	225	Intron 2
Exon 3	226	355	Intron 3
Exon 4	356	468	Intron 4
Exon 5	469	662	Intron 5
Exon 6	663	865	Intron 6
Exon 7	866	1033	Intron 7
Exon 8	1034	1090	Intron 8
Exon 9	1091	1224	Intron 9
Exon 10	1225	1299	Intron 10
Exon 11	1300	1458	Intron 11
Exon 12	1459	1587	Intron 12
Exon 13	1588	1687	Intron 13
Exon 14	1688	1909	Intron 14
Exon 15	1910	1963	Intron 15
Exon 16	1964	2064	Intron 16
Exon 17	2065	2211	Intron 17
Exon 18	2212	3432	

APP Gene Mutations.

Certain families are genetically predisposed to Alzheimer's disease, a condition referred to as familial Alzheimer's disease (FAD), through mutations resulting in an amino acid replacement at position 717 of the full length protein (Goate *et al.* (1991); Murrell *et al.* (1991); Chartier-Harlin *et al.* (1991)). These mutations co-segregate with the disease within the families. For example, Murrell *et al.* (1991) described a specific mutation found in exon 17 (which Murrell *et al.* refers to as exon 15) where the valine of position 717 is replaced by phenylalanine.

Another FAD mutant form contains a change in amino acids at positions 670 and 671 of the full length protein (Mullan *et al.* (1992)). In one form of this mutation, the lysine at position 670 is replaced by asparagine and the methionine at position 671 is replaced by leucine. The effect of this mutation is to increase the production of Aβ in cultured cells approximately 7-fold (Citron *et al.*, *Nature* 360: 672-674 (1992); Lai *et al.*, *Science* 259:514-516 (1993)). Replacement of the methionine at position 671 with leucine by itself has also been shown to increase production of Aβ.

Additional mutations in APP at amino acids 669, 670, and 671 have been shown to reduce the amount of Aβ processed from APP (Citron *et al.*, *Neuron* 14:661-670 (1995)). The APP construct with Val at amino acid 690 produces an increased amount of a truncated form of Aβ.

APP expression clones can be constructed that bear a mutation at amino acid 669, 670, 671, 690, 692, or 717 of the full length protein. The mutations from Lys to Asn and from Met to Leu at amino acids 670 and 671, respectively, are sometimes referred to as the Swedish mutation. Additional mutations can also be introduced at amino acids 669, 670, or 671 which either increase or reduce the amount of Aβ processed from APP. Mutations at these amino acids in any APP clone or transgene can be created by site-directed mutagenesis (Vincent *et al.*, *Genes & Devel.* 3:334-347 (1989)), or, once made, can be incorporated into other constructs using standard genetic engineering techniques. Some mutations at amino acid 717 are sometimes referred to as the Hardy mutation. Such mutations can include conversion of the wild-type Val717 codon to a codon for Ile, Phe, Gly, Tyr, Leu, Ala, Pro, Trp, Met, Ser, Thr, Asn, or Gln. A preferred substitution for Val717 is Phe. These mutations predispose individuals expressing the mutant proteins to develop Alzheimer's disease. It is believed that the mutations affect the expression and/or processing of APP, shifting the balance toward Alzheimer's pathology. Mutations at amino acid 669 can include conversion of the wild-type Val669 codon to a codon for Trp, or deletion of the codon. Mutations at amino acid 670 can include conversion of the wild-type Lys670 codon to a codon for Asn or Glu, or deletion of the codon. Mutations at amino acid 671 can include conversion of the wild-type Met671 codon to a codon for Leu, Val, Lys, Tyr, Glu, or Ile, or deletion of the codon. A preferred substitution for Lys670 is Asn, and a preferred substitution for Met671 is Leu. These mutations predispose individuals expressing the mutant proteins to develop Alzheimer's disease. The other listed mutations to amino acids 669, 670, and 671 are known to reduce the amount of Aβ processed from APP (Citron *et al.* (1995)). It is believed that

these mutations affect processing of APP leading to a change in Aβ production.

Truncated forms of APP can also be expressed from transgene constructs. For example, APP cDNA truncated to encode amino acids 646 to 770 of APP. The APP cDNA construct truncated to encode amino acids 646 to 770 of APP, and operatively linked to the PDGF-B promoter, is referred to as PDAPPc125.

Nucleic Acid Constructs Encoding Aβ-containing Proteins.

Constructs for use in transgenic animals include a promoter for expression of the construct in a mammalian cell and a region encoding a protein that includes all or a contiguous portion of one of the three forms of APP: APP695, APP751, or APP770, with or without specific amino acid mutations as described herein. It is preferred that protein encoded is an Aβ-containing protein. As used herein, an Aβ-containing protein is a protein that includes all or a contiguous portion of one of the three forms of APP: APP695, APP751, or APP770, with or without specific amino acid mutations as described herein, where the protein includes all or a portion of amino acids 672 to 714 of human APP. Preferred Aβ-containing proteins include amino acids 672 to 714 of human APP. Preferred forms of such Aβ-containing proteins include all or a contiguous portion of APP770, APP770 bearing a mutation in amino acid 669, 670, 671, 690, 692, and/or 717, APP751, APP751 bearing a mutation in amino acid 669, 670, 671, 690, 692, and/or 717, APP695, and APP695 bearing a mutation in amino acid 669, 670, 671, 690, 692, and/or 717, where each of these Aβ-containing proteins includes amino acids 672 to 714 of human APP.

Preferred forms of the above Aβ-containing proteins are APP770; APP770 bearing a mutation in the codon encoding one or more amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, 717; APP751; APP751 bearing a mutation in the codon encoding one or more amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, 717; APP695; APP695 bearing a mutation in the codon encoding one or more amino acids selected from the group consisting of

amino acid 669, 670, 671, 690, 692, 717; a protein consisting of amino acids 646 to 770 of APP; a protein consisting of amino acids 670 to 770 of APP; a protein consisting of amino acids 672 to 770 of APP; and a protein consisting of amino acids 672 to 714 of APP.

In the constructs disclosed herein, the DNA encoding the Aβ-containing protein can be cDNA or a cDNA/genomic DNA hybrid, wherein the cDNA/genomic DNA hybrid includes at least one APP intron sequence wherein the intron sequence is sufficient for splicing.

Preferred constructs contain DNA encoding APP770; DNA encoding APP770 bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a fragment of DNA encoding APP770 which encodes an amino acid sequence comprising amino acids 672 to 714 of APP770; DNA encoding APP751; DNA encoding APP751 bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a fragment of DNA encoding APP751 which encodes an amino acid sequence comprising amino acids 672 to 714 of APP770; DNA encoding APP695; DNA encoding APP695 bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a fragment of DNA encoding APP695 which encodes an amino acid sequence comprising amino acids 672 to 714 of APP770; APP cDNA truncated to encode amino acids 646 to 770 of APP; a combination cDNA/genomic DNA hybrid APP gene construct; a combination cDNA/genomic DNA hybrid APP gene construct bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; or a combination cDNA/genomic DNA hybrid APP gene construct truncated at amino acid 671 or 685.

Preferred forms of such constructs are APP770 cDNA; APP770 cDNA bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a fragment of APP770 cDNA encoding an APP amino acid sequence, the amino acid sequence comprising amino acids 672 to 714 of APP770; APP751 cDNA; APP751

cDNA bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a fragment of APP751 cDNA encoding an APP amino acid sequence, the amino acid sequence comprising amino acids 672 to 714 of APP770; APP695 cDNA; APP695 cDNA bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations; a fragment of APP695 cDNA encoding an APP amino acid sequence, the amino acid sequence comprising amino acids 672 to 714 of APP770; APP cDNA truncated to encode amino acids 646 to 770 of APP; a combination cDNA/genomic DNA hybrid APP gene construct; a combination cDNA/genomic DNA hybrid APP gene construct bearing a mutation in the codon encoding amino acid 669, 670, 671, 690, 692, 717, and a combination of these mutations; and a combination cDNA/genomic DNA hybrid APP gene construct truncated at amino acid 671 or 685.

Construction of Transgenes.

Construction of various APP transgenes can be accomplished using any suitable genetic engineering technique, such as those described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, N.Y., 1989). Regions of APP clones that have been engineered or mutated can be interchanged by using convenient restriction enzyme sites present in APP cDNA clones. A *Nru*I site starts at position -5 (relative to the first nucleotide of the AUG initiator codon). A *Kpn*I and an *Asp*718 site both start at position 57 (these are isoschizomers leaving different sticky ends). A *Xcm*I site starts at position 836 and cuts at position 843. A *Sca*I site starts at position 1004. A *Xho*I site starts at position 1135. A *Bam*HI site starts at position 1554. A *Bgl*II site starts at position 1994. An *Eco*RI site starts at position 2020. A *Spe*I site starts at position 2583. Another *Eco*RI site starts at position 3076.

The clones bearing various portions of the human APP gene sequence shown in Figures 1 to 5 can be constructed in a common manner using standard genetic engineering techniques. For example, these clones can be constructed by first cloning the polyA addition signal from SV40 virus, as a

253 base pair *Bcl*I to *Bam*HI fragment (Reddy *et al.*, *Science* 200:494-502 (1978), into a modified vector from the pUC series. Next, the cDNA coding sequences (APP770, APP751, or APP695) can be inserted. Correct orientation and content of the fragments inserted can be determined through restriction endonuclease mapping and limited sequencing. The clones bearing various carboxy terminal portions of the human APP gene sequence shown in Figures 4 and 5 can be constructed through several steps in addition to those indicated above. For example, an APP770 cDNA clone (SEQ ID NO:5) can be digested with *Asp*718 which cleaves after nucleotide position 57. The resulting 5' extension is filled in using the Klenow enzyme (Sambrook *et al.* (1989)) and ligated to a hexanucleotide of the following sequence: AGATCT, the recognition site for *Bgl*II. After cleavage with *Bgl*II, which also cuts after position 1994, and re-ligation, the translational reading frame of the protein is preserved. The truncated protein thus encoded contains the leader sequence, followed by approximately 6 amino acids that precede the Aβ, followed by the Aβ, and the 56 terminal amino acids of APP. The clone in Figure 5 is created by converting the nucleotide at position 2138 to a T by site directed mutagenesis in the clone of Figure 4a, thus creating a termination codon directly following the last amino acid codon of the Aβ. APP cDNA clones naturally contain an *Nru*I site that cuts 2 nucleotides upstream from the initiator methionine codon. This site can be used for attachment of the different promoters used to complete each construct.

APP transgenes can also be constructed using PCR cloning techniques. Such techniques allow precise coupling of DNA fragments in the transgenes.

Combination cDNA/Genomic DNA Clones.

Endogenous APP expression results from transcription of precursor mRNA followed by alternative splicing to produce three main forms of APP. It is believed that this alternative splicing may be important in producing the pattern of APP expression involved in Alzheimer's disease. It is also believed that the presence of introns in expression constructs can influence the level and nature of expression by, for example, targeting precursor

mRNA to mRNA processing and transport pathways (Huang *et al.*, *Nucleic Acids Res.* 18:937-947 (1990)). Accordingly, transgenes combining cDNA and genomic DNA, which include intron sequences, are a preferred type of construct.

The RNA splicing mechanism requires only a few specific and well known consensus sequences. Such sequences have been identified in APP genomic DNA by Yoshikai *et al.* (1990). The disclosed transgenes can be constructed using one or more complete and intact intron sequences. However, it is preferred that the transgenes are constructed using truncated intron sequences that contain an effective amount of intron sequence to allow splicing. In general, truncated intron sequences that retain the splicing donor site, the splicing acceptor site, and the splicing branchpoint sequence will constitute an effective amount of an intron. The sufficiency of any truncated intron sequence can be determined by testing for the presence of correctly spliced mRNA in transgenic cells using methods described below.

Other intron sequences and splicing signals which are not derived from APP gene sequences may also be used in the transgene constructs. Such intron sequences will enhance expression of the transgene construct. A preferred heterologous intron is a hybrid between the adenovirus major late region first exon and intron junction and an IgG variable region splice acceptor. This hybrid intron can be constructed, for example, by joining the 162 bp *Pvu*II to *Hind*III fragment of the adenovirus major late region, containing 8 bp of the first exon and 145 bp of the first intron, and the 99 bp *Hind*III to *Pst*I fragment of the IgG variable region splice acceptor clone-6, as described by Bothwell *et al.*, *Cell* 24:625-637 (1981). A similar splice signal has been shown to enhance expression of a construct to which it was attached, as described by Manley *et al.*, *Nucleic Acids Res.* 18:937-947 (1990). It is preferred that the heterologous intron be placed between the promoter and the region encoding the APP.

A preferred APP combination cDNA/genomic expression clone includes an effective amount of introns 6, 7 and 8, as shown in Figure 6. Such a transgene can be constructed as follows. A preferred method of

construction is described in Example 5. A plasmid containing the cDNA portion of the clone can be constructed by first converting the *Taq*I site at position 860 in an APP770 cDNA clone to an *Xho*I site by site-directed mutagenesis. Cleavage of the resulting plasmid with *Xho*I cuts at the new *Xho*I site and a pre-existing *Xho*I site at position 1135, and releases the KPI and OX-2 coding sequence. The plasmid thus generated serves as the acceptor for the KPI and OX-2 alternative splicing cassette.

The alternative splicing cassette can be created through a series of cloning steps involving genomic DNA. First, the *Taq*I site at position 860 in a genomic clone containing exon 6 and the adjacent downstream intron can be converted to an *Xho*I site by site-directed mutagenesis. Cleavage of the resulting plasmid with *Xho*I cuts at the new *Xho*I site and an *Xho*I site within either intron 6 or 7. This fragment, containing a part of exon 6 and at least a part of adjacent intron 6, can then be cloned into the *Xho*I site in a plasmid vector. Second, a genomic clone containing exon 9 and the adjacent upstream genomic sequences is cleaved with *Xho*I, cleaving the clone at the *Xho*I site at position 1135 (position 910 using the numbering system of Kang *et al.* (1987)) and an *Xho*I site in either intron 7 or 8. This fragment, containing a part of exon 9 and at least a part of adjacent intron 8, can then be cloned into the *Xho*I site of another plasmid vector. These two exon/intron junction fragments can then be released from their respective plasmid vectors by cleavage with *Xho*I and either *Bam*HI or *Bgl*II, and cloned together into the *Xho*I site of another plasmid vector. It is preferred that the exon/intron junction fragments be excised with *Bam*HI. It is most preferable that BamHI sites are engineered in the intron portion of the exon/intron junction fragments prior to their excision. This allows the elimination of lengthy extraneous intron sequences from the cDNA/genomic clone.

The *Xho*I fragment resulting from cloning the two exon/intron junction fragments together can be cleaved with either *Bam*HI or *Bgl*II, depending on which enzyme was used for excision step above, and the genomic 6.8 kb *Bam*HI segment, containing the KPI and OX-2 coding region along with their flanking intron sequences, can be inserted. This fragment was identified by

Kitaguchi *et al.* (1988) using Southern blot analysis of *Bam*HI-digested lymphocyte DNA from one normal individual and eight Alzheimer's disease patients using a 212 bp *Taq*I-*Ava*I fragment, nucleotides 862 to 1,073, of APP770 cDNA as the hybridization probe. Genomic DNA clones containing the region of the 225 bp insert can be isolated, for example, from a human leukocyte DNA library using the 212 bp *Taq*I-*Ava*I fragment as a probe. In the genomic DNA, the 225 bp sequence is located in a 168 bp exon (exon 7) and a 57 bp exon (exon 8), separated by an intron of approximately 2.6 kb (intron 7), with both exons flanked by intron-exon consensus sequences. The exon 7 corresponds to nucleotides 866 to 1,033 of APP770, and the exon 8 to nucleotides 1,034 to 1,090. Exon 7 encodes the highly conserved region of the Kunitz-type protease inhibitor family domain.

After cleavage with *Xho*I, this alternative splicing cassette, containing both exon and intron sequences, can then be excised by cleavage with XhoI and inserted into the *Xho*I site of the modified APP770 cDNA plasmid (the acceptor plasmid) constructed above. These cloning steps generate a combination cDNA/genomic expression clone that allows cells in a transgenic animal to regulate the inclusion of the KPI and OX-2 domains by a natural alternative splicing mechanism. An analogous gene bearing a mutation at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations, can be constructed either directly by *in vitro* mutagenesis. A mutation to amino acid 717 can also be made by using the mutated form of APP770 cDNA described above to construct an acceptor plasmid.

Promoters.

Different promoter sequences can be used to control expression of nucleotide sequences encoding Aβ-containing proteins. The ability to regulate expression of the gene encoding an Aβ-containing protein in transgenic animals is believed to be useful in evaluating the roles of the different APP gene products in AD. The ability to regulate expression of the gene encoding an Aβ-containing protein in cultured cells is believed to be useful in evaluating expression and processing of the different Aβ-containing gene products and may provide the basis for cell cultured drug screens. A

preferred promoter is the human platelet derived growth factor β (PDGF-B) chain gene promoter (Sasahara *et al.*, *Cell* 64:217-227 (1991)).

Preferred promoters for the disclosed APP constructs are those that, when operatively linked to the protein coding sequences, mediate expression of one or more of the following expression products to at least a specific level in brain tissue of a two to four month old animal transgenic for one of the disclosed APP constructs. The products and their expression levels are $A\beta_{tot}$ to a level of at least 30 ng/g (6.8 pmoles/g) brain tissue and preferably at least 40 ng/g (9.12 pmoles/g) brain tissue, $A\beta_{1-42}$ to a level of at least 8.5 ng/g (1.82 pmoles/g) brain tissue and preferably at least 11.5 ng/g (2.5 pmoles/g) brain tissue, full length APP (FLAPP) and APPα combined (FLAPP+APPα) to a level of at least 150 pmoles/g brain tissue, APPβ to a level of at least 42 pmoles/g brain tissue, and mRNA encoding human Aβ-containing protein to a level at least twice that of mRNA encoding the endogenous APP of the transgenic animal. $A\beta_{tot}$ is the total of all forms of Aβ. $A\beta_{1-42}$ is a form of Aβ having amino acids 1 to 42 of Aβ (corresponding to amino acids 672 to 714 of APP). FLAPP+APPα refers to APP forms containing the first 12 amino acids of the Aβ region (corresponding to amino acids 672 to 684 of APP). Thus, FLAPP+APPα represents a mixture of full length forms of APP and APP cleaved at the α-secretase site (Esch *et al.*, *Science* 248:1122-1124 (1990)). APPβ is APP cleaved at the β-secretase site (Seubert *et al.*, *Nature* 361:260-263 (1993)).

It is intended that the levels of expression described above refer to amounts of expression product present and are not limited to the specific units of measure used above. Thus, an expression level can be measured, for example, in moles per gram of tissue, grams per grams of tissue, moles per volume of tissue, and in grams per volume of tissue. The equivalence of these units of measure to the measures listed above can be determined using known conversion methods.

The levels of expression described above need not occur in all brain tissues. Thus, a promoter is considered preferred if at least one of the levels of expression described above occurs in at least one type of brain tissue.

Where expression is tissue-specific, it is understood that if the expression level is sufficient in the specific brain tissue, the promoter is considered preferred even though the expression level in brain tissue as a whole may not, and need not, reach a threshold level. It is preferred that this level of expression is observed in hippocampal and/or cortical brain tissue. The promoter can mediate expression of the above expression products to the levels described above either constitutively or by induction. Induction can be accomplished by, for example, administration of an activator molecule, by heat, or by expression of a protein activator of transcription for the promoter operatively linked to the gene encoding an Aβ-containing protein. Many inducible expression systems which would be suitable for this purpose are known to those of skill in the art.

It is preferred that, in making the above measurements, the brain tissue is prepared by the following method. A brain from a transgenic test animal is dissected and the tissue is kept on ice throughout the homogenization procedure except as noted. The brain tissue is homogenized in 10 volumes (w/v) of 5 M guanidine-HCl, 50 mM Tris-HCl, pH 8.5. The sample is then gently mixed for 2 to 4 hours at room temperature. Homogenates are then diluted 1:10 in cold casein buffer #1 (0.25% casein/phosphate buffered saline (PBS) 0.05% sodium azide, pH 7.4, 1X protease inhibitor cocktail) for a final 0.5 M guanidine concentration and kept on ice. 100X protease inhibitor cocktail is composed of 2 mg/ml aprotinin, 0.5 M EDTA, pH 8.0, 1 mg/ml leupeptin. Diluted homogenates are then spun in an Eppendorf microfuge at 14,000 rpm for 20 minutes at 4°C. If further dilutions are required, they can be made with cold guanidine buffer #2 (1 part guanidine buffer #1 to 9 parts casein buffer #1).

It is preferred that the following assay be used to identify preferred promoters for their ability to mediate expression of Aβ to the levels described above. Antibody 266 (Seubert *et al.*, *Nature* 359:325-327 (1992)) is dissolved at 10 μg/ml in buffer (0.23 g/L NaH_2PO_4-H_2O, 26.2 g/L $NaHPO_4$-$7H_2O$, 1 g/L sodium azide adjusted to pH 7.4) and 100 μl/well is coated onto 96-well immunoassay plates (Costar) and allowed to bind overnight. The

plate is then aspirated and blocked for at least 1 hour with a 0.25% human serum albumin solution in 25 g/L sucrose, 10.8 g/L Na_2HPO_4-$7H_2O$, 1.0 g/L NaH_2PO_4-H_2O, 0.5 g/L sodium azide adjusted to pH 7.4. The 266 coated plate is then washed 1X with wash buffer (PBS/0.05% Tween 20) using a Skatron plate washer. 100 µl/well of Aβ1-40 standards and brain tissue samples are added to the plate in triplicate and incubated overnight at 4°C. Aβ1-40 standards are made from 0.0156, 0.0312, 0.0625, 0.125, 0.250, 0.500, and 1.000 µg/ml stocks in DMSO stored at -40° C as well as a DMSO only control for background determination. Aβ standards consist of 1:100 dilution of each standard into guanidine buffer #3 (1 part BSA buffer to 9 parts guanidine buffer #1) followed by a 1:10 dilution into casein buffer #1 (Note: the final Aβ concentration range is 15.6 to 1000 pg/ml and the final guanidine concentration is 0.5 M). BSA buffer consists of 1% bovine serum albumin (BSA, immunoglobulin-free)/PBS/0.05% sodium azide. The plates and casein buffer #2 (0.25% casein/PBS/0.05% Tween 20/pH 7.4) are then brought to room temperature (RT). The plates are then washed 3X with wash buffer. Next, 100 µl/well of 3D6-biotin at 0.5 µg/ml in casein buffer #2 is added to each well and incubated at 1 hour at RT.

Monoclonal antibody 3D6 was raised against the synthetic peptide DAEFRGGC (SEQ ID NO:10) which was conjugated through the cysteine to sheep anti-mouse immunoglobulin. The antibody does not recognize secreted APP but does recognize species that begin at Aβ position 1 (Asp). For biotinylating 3D6, follow Pierce's NHS-Biotin protocol for labeling IgG (cat. #20217X) except use 100 mM sodium bicarbonate, pH 8.5 and 24 mg NHS-biotin per ml of DMSO.

The plates are then again washed 3X with wash buffer. Then, 100 µl/well of horseradish peroxidase (HRP)-avidin (Vector Labs, cat. # A-2004) diluted 1:4000 in casein buffer #2 is added to each well and incubated for 1 hour at RT. The plates are washed 4X with wash buffer and then 100 µl/well of TMB substrate (Slow TMB-ELISA (Pierce cat. # 34024)) at RT is added to each well and incubated for 15 minutes at RT. Finally, 25 µl/well

of 2 N H_2SO_4 is added to each well to stop the enzymatic reaction, and the plate is read at 450 nm to 650 nm using the Molecular Devices Vmax reader.

It is preferred that the relative levels of mRNA encoding human Aβ-containing protein mRNA encoding the endogenous APP of the transgenic animal be measured in the manner described by Bordonaro *et al.*, *Biotechniques* 16:428-430 (1994), and Rockenstein *et al.*, *J. Biol. Chem.* 270:28257-28267 (1995). Preferred methods for measuring the expression level of Aβ_{1-42}, FLAPP+APPα, and APPβ are described in Example 8.

Yeast Artificial Chromosomes.

The constructs shown in Figure 7 can be constructed as follows. Large segments of human genomic DNA, when cloned into certain vectors, can be propagated as autonomously-replicating units in the yeast cell. Such vector-borne segments are referred to as yeast artificial chromosomes (YAC; Burke *et al. Science* 236:806 (1987)). A human YAC library is commercially available (Clontech, Palo Alto, CA) with an average insert size of 250,000 base pairs (range of 180,000 to 500,000 base pairs). A YAC clone of the Alzheimer's gene can be directly isolated by screening the library with the human APP770 cDNA. The inclusion of all of the essential gene regions in the clone can be confirmed by PCR analysis.

The YAC-APP clone, shown in Figure 7a, can be established in embryonic stem (ES) cells by selecting for neomycin resistance encoded by the YAC vector. ES cells bearing the YAC-APP clone can be used to produce transgenic mice by established methods described below under "Transgenic Mice" and "Embryonic Stem Cell Methods". The YAC-APP gene bearing a mutation at amino acid 717 (Figure 7b) can be produced through the generation of a YAC library using genomic DNA from a person affected by a mutation at amino acid 717. Such a clone can be identified and established in ES cells as described above.

Genetic Alteration of the Mouse APP Gene.

The nucleotide sequence homology between the human and murine Alzheimer's protein genes is approximately 85%. Within the Aβ-coding region, there are three amino acid differences between the two sequences.

Amino acids Lys 670, Met671, and Val717,which can be mutated to alter APP processing, are conserved between mouse, rat, and man. Wild-type rodents do not develop Alzheimer's disease nor do they develop deposits or plaques in their central nervous system (CNS) analogous to those present in human Alzheimer's patients. Therefore, it is possible that the human but not the rodent form of Aβ is capable of causing disease. Homologous recombination (Capecchi, *Science* 244:1288-1292 (1989)) can be used to convert the mouse Alzheimer's gene *in situ* to a gene encoding the human Aβ by gene replacement. This recombination is directed to a site downstream from the KPI and OX-2 domains, for example, within exon 9, so that the natural alternative splicing mechanisms appropriate to all cells within the transgenic animal can be employed in expressing the final gene product.

Both wild-type (Figure 8a) and mutant (Figure 8b) forms of human cDNA can be used to produce transgenic models expressing either the wild-type or mutant forms of APP. The recombination vector can be constructed from a human APP cDNA (APP695, APP751, or APP770 form), either wild-type, mutant at amino acid 669, 670, 671, 690, 692, 717, or a combination of these mutations. Cleavage of the recombination vector, for example, at the *Xho*I site within exon 9, promotes homologous recombination within the directly adjacent sequences (Capecchi (1989)). The endogenous APP gene resulting from this event would be normal up to the point of recombination, within exon 9 in this example, and would consist of the human cDNA sequence thereafter.

Preparation of Constructs for Transfections and Microinjections.

DNA clones for microinjection are cleaved with enzymes appropriate for removing the bacterial plasmid sequences, such as *Sal*I and *Not*I, and the DNA fragments electrophoresed on 1% agarose gels in TBE buffer (Sambrook *et al.* (1989)). The DNA bands are visualized by staining with ethidium bromide, and the band containing the APP expression sequences is excised. The excised band is then placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with phenol-chloroform (1:1), and precipitated by two volumes of

ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D™ column. The column is first primed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column for three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml of high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 μg/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA. Other methods for purification of DNA for microinjection are also described in Hogan *et al.*, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986); in Palmiter *et al.*, *Nature* 300:611 (1982); in *The Qiagenologist, Application Protocols*, 3rd edition, published by Qiagen, Inc., Chatsworth, CA.; and in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Construction of Transgenic Animals.

A. Animal Sources.

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), and Harlan Sprague Dawley (Indianapolis, IN). Many strains are suitable, but Swiss Webster (Taconic) female mice are preferred for embryo retrieval and transfer. B6D2F_1 (Taconic) males can be used for mating and vasectomized Swiss Webster studs can be used to stimulate pseudopregnancy. Vasectomized mice and rats can be obtained from the supplier.

B. Microinjection Procedures.

The procedures for manipulation of the rodent embryo and for microinjection of DNA are described in detail in Hogan *et al.*, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor,

NY, 1986), the teachings of which are generally known and are incorporated herein.

C. Transgenic Mice.

Female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5°C incubator with a humidified atmosphere at 5% CO_2, 95% air until the time of injection. Embryos can be implanted at the two cell stage.

Randomly cycling adult female mice are paired with vasectomized males. Swiss Webster or other comparable strains can be used for this purpose. Recipient females are mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5% avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is then torn with watchmakers forceps. Embryos to be transferred are placed in DPBS (Dulbecco's phosphate buffered saline) and in the tip of a transfer pipet (about 10 to 12 embryos). The pipet tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures.

D. Transgenic Rats.

The procedure for generating transgenic rats is similar to that of mice (Hammer *et al.*, *Cell* 63:1099-112 (1990)). Thirty day-old female rats are given a subcutaneous injection of 20 IU of PMSG (0.1 cc) and 48 hours later

each female placed with a proven male. At the same time, 40-80 day old females are placed in cages with vasectomized males. These will provide the foster mothers for embryo transfer. The next morning females are checked for vaginal plugs. Females who have mated with vasectomized males are held aside until the time of transfer. Donor females that have mated are sacrificed (CO_2 asphyxiation) and their oviducts removed, placed in DPBS (Dulbecco's phosphate buffered saline) with 0.5% BSA and the embryos collected. Cumulus cells surrounding the embryos are removed with hyaluronidase (1 mg/ml). The embryos are then washed and placed in EBSS (Earle's balanced salt solution) containing 0.5% BSA in a 37.5°C incubator until the time of microinjection.

Once the embryos are injected, the live embryos are moved to DPBS for transfer into foster mothers. The foster mothers are anesthetized with ketamine (40 mg/kg, ip) and xylazine (5 mg/kg, ip). A dorsal midline incision is made through the skin and the ovary and oviduct are exposed by an incision through the muscle layer directly over the ovary. The ovarian bursa is torn, the embryos are picked up into the transfer pipet, and the tip of the transfer pipet is inserted into the infundibulum. Approximately 10 to 12 embryos are transferred into each rat oviduct through the infundibulum. The incision is then closed with sutures, and the foster mothers are housed singly.

E. Embryonic Stem (ES) Cell Methods.

1. Introduction of cDNA into ES cells.

Methods for the culturing of ES cells and the subsequent production of transgenic animals, the introduction of DNA into ES cells by a variety of methods such as electroporation, calcium phosphate/DNA precipitation, and direct injection are described in detail in *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach*, ed. E.J. Robertson, (IRL Press 1987), the teachings of which are generally known and are incorporated herein. Selection of the desired clone of transgene-containing ES cells can be accomplished through one of several means. For random gene integration, an APP clone is co-precipitated with a gene encoding neomycin resistance. Transfection is carried out by one of several methods described in detail in

Lovell-Badge, in *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach*, ed. E.J. Robertson, (IRL Press 1987), or in Potter *et al.*, *Proc. Natl. Acad. Sci. USA* 81:7161 (1984). Lipofection can be performed using reagents such as provided in commercially available kits, for example DOTAP (Boehringer-Mannheim) or lipofectin (BRL). Calcium phosphate/DNA precipitation, lipofection, direct injection, and electroporation are the preferred methods. In these procedures, 0.5×10^6 ES cells are plated into tissue culture dishes and transfected with a mixture of the linearized APP clone and 1 mg of pSV2neo DNA (Southern and Berg, *J. Mol. Appl. Gen.* 1:327-341 (1982)) precipitated in the presence of 50 mg lipofectin (BRL) in a final volume of 100 μl. The cells are fed with selection medium containing 10% fetal bovine serum in DMEM supplemented with G418 (between 200 and 500 μg/ml). Colonies of cells resistant to G418 are isolated using cloning rings and expanded. DNA is extracted from drug resistant clones and Southern blots using an APP770 cDNA probe can be used to identify those clones carrying the APP sequences. PCR detection methods may also used to identify the clones of interest.

DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination, described by Capecchi (1989). Direct injection results in a high efficiency of integration. Desired clones can be identified through PCR of DNA prepared from pools of injected ES cells. Positive cells within the pools can be identified by PCR subsequent to cell cloning (Zimmer and Gruss, *Nature* 338:150-153 (1989). DNA introduction by electroporation is less efficient and requires a selection step. Methods for positive selection of the recombination event (for example, neo resistance) and dual positive-negative selection (for example, neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Joyner *et al.*, *Nature* 338:153-156 (1989), and Capecchi (1989), the teachings of which are generally known and are incorporated herein.

2. Embryo Recovery and ES cell Injection.

Naturally cycling or superovulated female mice mated with males can be used to harvest embryos for the implantation of ES cells. It is desirable to use the C57BL/6 strain for this purpose when using mice. Embryos of the appropriate age are recovered approximately 3.5 days after successful mating. Mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are flushed from excised uterine horns and placed in Dulbecco's modified essential medium plus 10% calf serum for injection with ES cells. Approximately 10 to 20 ES cells are injected into blastocysts using a glass microneedle with an internal diameter of approximately 20 μm.

3. Transfer of Embryos to Pseudopregnant Females.

Randomly cycling adult female mice are paired with vasectomized males. Mouse strains such as Swiss Webster, ICR or others can be used for this purpose. Recipient females are mated such that they will be at 2.5 to 3.5 days post-mating when required for implantation with blastocysts containing ES cells. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5% avertin per gram of body weight. The ovaries are exposed by making an incision in the body wall directly over the oviduct and the ovary and uterus are externalized. A hole is made in the uterine horn with a 25 gauge needle through which the blastocysts are transferred. After the transfer, the ovary and uterus are pushed back into the body and the incision is closed by two sutures. This procedure is repeated on the opposite side if additional transfers are to be made.

Identification, Characterization, and Utilization of Transgenic Mice and Rats.

Transgenic rodents can be identified by analyzing their DNA. For this purpose, tail samples (1 to 2 cm) can be removed from three week old animals. DNA from these or other samples can then be prepared and analyzed by Southern blot, PCR, or slot blot to detect transgenic founder (F_0) animals and their progeny (F_1 and F_2).

A. Pathological Studies.

The various F_0, F_1, and F_2 animals that carry a transgene can be analyzed by immunohistology for evidence of Aβ deposition, expression of APP or APP cleavage products, neuronal or neuritic abnormalities, and inflammatory responses in the brain. Brains of mice and rats from each transgenic line are fixed and then sectioned. Sections are stained with antibodies reactive with the APP and/or the Aβ. Secondary antibodies conjugated with fluorescein, rhodamine, horse radish peroxidase, or alkaline phosphatase are used to detect the primary antibody. These methods permit identification of amyloid plaques and other pathological lesions in specific areas of the brain. Plaques ranging in size from 9 to >50 μm characteristically occur in the brains of AD patients in the cerebral cortex, but also may be observed in deeper grey matter including the amygdaloid nucleus, corpus striatum and diencephalon. Sections can also be stained with other antibodies diagnostic of Alzheimer's plaques, recognizing antigens such as APP, Alz-50, tau, A2B5, neurofilaments, synaptophysin, MAP-2, ubiquitin, complement, neuron-specific enolase, and others that are characteristic of Alzheimer's pathology (Wolozin *et al.*, *Science* 232:648 (1986); Hardy and Allsop, *Trends in Pharm. Sci.* 12:383-388 (1991); Selkoe, *Ann. Rev. Neurosci.* 12:463-490 (1989); Arai *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2249-2253 (1990); Majocha *et al.*, *Amer. Assoc. Neuropathology Abs* 99:22 (1988); Masters *et al.*, *Proc. Natl. Acad. Sci.* 82:4245-4249 (1985); Majocha *et al.*, *Can J Biochem Cell Biol* 63:577-584 (1985)). Staining with thioflavin S and Congo Red can also be carried out to analyze the presence of amyloid and co-localization of Aβ deposits within neuritic plaques and NFTs.

B. Analysis of APP and Aβ Expression.

1. mRNA.

Messenger RNA can be isolated by the acid guanidinium thiocyanate-phenol:chloroform extraction method (Chomaczynski and Sacchi, *Anal Biochem* 162:156-159 (1987)) from cell lines and tissues of transgenic animals to determine expression levels by Northern blots, RNAse and nuclease protection assays.

2. Protein.

APP, Aβ, and other fragments of APP can and have been detected by using polyclonal and monoclonal antibodies that are specific to the APP extra-cytoplasmic domain, Aβ region, $A\beta_{1-42}$, $A\beta_{1-40}$, APPβ, FLAPP+APPα, and C-terminus of APP. A variety of antibodies that are human sequence specific, such as 10D5 and 6C6, are very useful for this purpose (Games *et al.* (1995)).

3. Western Blot Analysis.

Protein fractions can be isolated from tissue homogenates and cell lysates and subjected to Western blot analysis as described by, for example, Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor, NY, 1988); Brown *et al.*, *J. Neurochem.* 40:299-308 (1983); and Tate-Ostroff *et al.*, *Proc Natl Acad Sci* 86:745-749 (1989).

Briefly, the protein fractions are denatured in Laemmli sample buffer and electrophoresed on SDS-Polyacrylamide gels. The proteins are then transferred to nitrocellulose filters by electroblotting. The filters are blocked, incubated with primary antibodies, and finally reacted with enzyme conjugated secondary antibodies. Subsequent incubation with the appropriate chromogenic substrate reveals the position of APP derived proteins.

C. Pathological and Behavioral Studies.

1. Pathological Studies.

Immunohistology and thioflavin S staining are conducted as described elsewhere herein.

***In situ* Hybridizations:** Radioactive or enzymatically labeled nucleic acid probes can be used to detect mRNA *in situ*. The probes are degraded or prepared to be approximately 100 nucleotides in length for better penetration of cells. The hybridization procedure of Chou *et al.*, *J. Psych. Res.* 24:27-50 (1990), for fixed and paraffin embedded samples is briefly described below although similar procedures can be employed with samples sectioned as frozen material. Paraffin slides for *in situ* hybridization are dewaxed in xylene and rehydrated in a graded series of ethanols and finally rinsed in phosphate buffered saline (PBS). The sections are post-fixed in fresh 4%

paraformaldehyde. The slides are washed with PBS twice for 5 minutes to remove paraformaldehyde. Then the sections are permeabilized by treatment with a 20 μg/ml proteinase K solution. The sections are re-fixed in 4% paraformaldehyde, and basic molecules that could give rise to background probe binding are acetylated in a 0.1 M triethanolamine, 0.3 M acetic anhydride solution for 10 minutes. The slides are washed in PBS, then dehydrated in a graded series of ethanols and air dried. Sections are hybridized with antisense probe, using sense probe as a control. After appropriate washing, bound radioactive probes are detected by autoradiography or enzymatically labeled probes are detected through reaction with the appropriate chromogenic substrates.

2. Behavioral Studies.

Behavioral tests designed to assess learning and memory deficits are employed. An example of such as test is the Morris water maze (Morris, *Learn Motivat.* 12:239-260 (1981)). In this procedure, the animal is placed in a circular pool filled with water, with an escape platform submerged just below the surface of the water. A visible marker is placed on the platform so that the animal can find it by navigating toward a proximal visual cue. Alternatively, a more complex form of the test in which there are no local cues to mark the platform's location will be given to the animals. In this form, the animal must learn the platform's location relative to distal visual cues, and can be used to assess both reference and working memory. A learning deficit in the water maze has been demonstrated with PDAPP transgenic mice. An example of behavioral analysis for assessing the effect of transgenic expression of Aβ-containing proteins is described in Example 9.

Operant behavior studies of memory function: Memory function of the disclosed transgenic animals can be assessed by testing memory-related feeding behavior (Dunnett, "Operant delayed matching and non-matching position in rats" in Behavioral Neuroscience, Volume I: A Practical Approach (Sagal, ed., IRL Press, N.Y., 1993) pages 123-136; Zornetzen, *Behav. Neur. Biol.* 36:49-60 (1982)). Transgenic and non-transgenic mice, are trained to earn food rewards in a two component operant procedure. One

component features a delayed spatial alternation schedule. Under this schedule, the mouse must remember over a variable time delay which lever it has pressed in the previous trial so that it can earn a reward by pressing the alternate lever on the current trial. This provides a measure of the animal's recent or "working" memory. The second component features a discrimination spatial alternation schedule. Under this schedule, the mouse earns a reward by pressing whatever lever is illuminated. This discrimination behavior is an example of reference memory. These two groups of mice, transgenic and non-transgenic, can be chronically studied over time, for example, from 3 months of age until the end of their useful life span, in order to assess the development of sensitivity to cholinergic antagonists and behavioral impairment on these memory tasks. It is expected that the disclosed transgenic mice will model the cognitive deficits of Alzheimer's disease with enhanced sensitivity to the memory-disrupting effects of cholinergic antagonists and impairment on "working" and reference memory tasks. Dose-response challenges with the cholinergic antagonist can be conducted at various ages. These memory behavioral tests can also be used to compare the effect of compounds on the behavioral impairment of the disclosed transgenic animals. In this case, the two groups of mice are transgenic mice to which a test compound is administered and transgenic mice to which the compound is not administered.

Emotional reactivity and object recognition: Various functions of the disclosed transgenic animals can be assessed by testing locomotor activity, emotional reactivity to a novel environment or to novel objects, and object recognition. A first set of assessments are performed in the same animals at different ages (each animal is its own control) in order to test their performance in terms of locomotor activity, emotional reactivity to a novel environment or to novel objects, and object recognition, a form of memory which is severely impaired in AD patients. On the first day, transgenic and non-transgenic control mice are individually placed in a square open field with a central platform. For 30 minutes, horizontal and vertical activity, and crossings of the platform, are recorded by blocks of 5 minutes for each

animal. On the second day, each animal is submitted to two trials with an intertrial of 1 hour. On the first trial, two identical objects are placed in the open field and the animal is allowed 3 minutes of exploration. On the second trial, one of the objects is replaced by a new object and the time spent by the animal in exploring the familiar and novel object is recorded during the next 3 minutes (Ennaceur and Delacour, *Behav. Brain Res.* 31:47-59 (1988)). Animals are then tested for neophobic behavior, which is considered as an index of anxiety, in a free exploration situation, in which animals are given the opportunity to move freely between a familiar and a novel environment.

Thereafter, the same animals are submitted to various learning tasks to investigate their learning and memory capacities. They are first tested for spatial recognition memory in a T-maze delayed alternation task at 6 hour and 24 hour delays. This form of memory has been shown to be very sensitive to hippocampal damage. One half of the animals of each group is then trained in a positively reinforced lever-press task as described above. This can be used to measure post training improvement in performance of the animals, which has been shown to involve hippocampal activation. The other half of the animals is trained in spatial discrimination in an 8 arm radial maze (Oltons and Samuelson, *J. Exp. Psychol. [Animal Behav.]* 2:97-116 (1976)) in order to evaluate working and reference memory and to analyze their strategies (angle preference), which give a better index of memory capacities. The animals trained and tested in the bar-lever press task at 2 to 3 months old can be trained and tested in radial maze at 9 to 10 months old, and vice-versa. A working memory deficit has been demonstrated in PDAPP transgenic mice in the radial arm maze.

Two additional groups can also be submitted to the same behavioral tests as above at 9 to 10 months old in order to determine whether behavioral screening performed at 2 to 3 months old influenced further learning and memory capacities.

These memory behavioral tests can also be used to compare the effect of compounds on the behavioral impairment of the disclosed transgenic animals. In this case, the two groups of mice are transgenic mice to which a

test compound is administered, and transgenic mice to which the compound is not administered.

The procedures applied to test transgenic mice are similar for transgenic rats.

D. Preferred Characteristics.

The above phenotypic characteristics of the disclosed transgenic animals can be used to identify those forms of the disclosed transgenic animals that are preferred as animal models. Additional phenotypic characteristics, and assays for measuring these characteristics, that can also be used to identify those forms of the disclosed transgenic animals that are preferred as animal models, are described in Example 6. These characteristics are preferably those that are similar to phenotypic characteristics observed in Alzheimer's disease. APP and Aβ markers which are also useful for identifying those forms of the disclosed transgenic animals that are preferred as animal models are described below. Any or all of the these markers or phenotypic characteristics can be used either alone or in combination to identify preferred forms of the disclosed transgenic animals. For example, the presence of plaques in brain tissue that can be stained with Congo red is a phenotypic characteristic which can identify a disclosed transgenic animal as preferred. It is intended that the levels of expression of certain APP-related proteins present in preferred transgenic animals (discussed above) is an independent characteristic for identifying preferred transgenic animals. Thus, the most preferred transgenic animals will exhibit both a disclosed expression level for one or more of the APP-related proteins and one or more of the phenotypic characteristics discussed above. Especially preferred phenotypic characteristics (the presence of which identifies the animal as a preferred transgenic animal) are the presence of amyloid plaques that can be stained with Congo Red (Kelly (1984)), the presence of extracellular amyloid fibrils as identified by electron microscopy by 12 months of age, and the presence of type I dystrophic neurites as identified by electron microscopy by 12 months of age (composed of spherical neurites that contain synaptic proteins and APP; Dickson *et al.*, *Am*

J Pathol 132:86-101 (1988); Dickson *et al.*, *Acta Neuropath.* 79:486-493 (1990); Masliah *et al.*, *J Neuropathol Exp Neurol* 52:135-142 (1993); Masliah *et al.*, *Acta Neuropathol* 87:135-142 (1994); Wang and Munoz, *J Neuropathol Exp Neurol* 54:548-556 (1995)). Examples of the detection of these characteristics is provided in Example 6. It is most preferred that the transgenic animals have amyloid plaques that can be stained with Congo Red as of 14 months of age.

Screening of Compounds for Treatment of Alzheimer's Disease.

The transgenic animals, or animal cells derived from transgenic animals, can be used to screen compounds for a potential effect in the treatment of Alzheimer's disease using standard methodology. In such AD screening assays, the compound is administered to the animals, or introduced into the culture media of cells derived from these animals, over a period of time and in various dosages, then the animals or animal cells are examined for alterations in APP expression or processing, expression levels or localization of other AD markers, histopathology, and/or, in the case of animals, behavior using the procedures described above and in the examples below. In general, any improvement in behavioral tests, alteration in AD-associated markers, reduction in the severity of AD-related histopathology, reduction in the expression of Aβ or APP cleavage products, and/or changes in the presence, absence or levels of other compounds that are correlated with AD which are observed in treated animals, relative to untreated animals, is indicative of a compound useful for treating Alzheimer's disease. The specific proteins, and the encoding transcripts, the enzymatic or biochemical activity, and/or histopathology of those proteins, that are associated with and characteristic of AD are referred to herein as markers. Expression or localization of these markers characteristic of AD has either been detected, or is expected to be present, in the disclosed transgenic animals. These markers can be measured or detected, and those measurements compared between treated and untreated transgenic animals to determine the effect of a tested compound.

Markers useful for AD screening assays are selected based on detectable changes in these markers that are associated with AD. Many such markers have been identified in AD and have either been detected in the disclosed transgenic animals or are expected to be present in these animals. These markers fall into several categories based on their nature, location, or function. Preferred examples of markers useful in AD screening assays are described below, group as Aβ-related markers, plaque-related markers, cytoskeletal and neuritic markers, inflammatory markers, and neuronal and neurotransmitter-related markers.

A. Aβ-related Markers.

Expression of the various forms of APP and Aβ can be directly measured and compared in treated and untreated transgenic animals both by immunohistochemistry and by quantitative ELISA measurements as described above and in the examples. Currently, it is known that two forms of APP products are found, APP and Aβ (Haass and Selkoe, *Cell* 75:1039-1042 (1993)). They have been shown to be intrinsically associated with the pathology of AD in a time dependent manner. Therefore, preferred assays compare age-related changes in APP and Aβ expression in the transgenic mice. As described in Example 6, increases in Aβ have been demonstrated during aging of the PDAPP mouse.

Preferred targets for assay measurement are Aβ markers known to increase in individuals with Alzheimer's disease are total Aβ ($A\beta_{tot}$), Aβ 1-42 ($A\beta_{1-42}$; Aβ with amino acids 1-42), $A\beta_{1-40}$ (Aβ with amino acids 1-40), Aβ N3(pE) ($A\beta_{N3}(pE)$); Aβ X-42 ($A\beta_{X-42}$; Aβ forms ending at amino acid 42); Aβ X-40 ($A\beta_{X-40}$; Aβ forms ending at amino acid 40); insoluble Aβ ($A\beta_{Insoluble}$); and soluble Aβ ($A\beta_{Soluble}$; Kuo *et al.*, *J. Biol. Chem.* 271(8):4077-4081 (1996)). $A\beta_{N3}(pE)$ has pyroglutamic acid at position 3 (Saido, *Neuron* 14:457-466 (1995)). $A\beta_{X-42}$ refers to any of the C-terminal forms of Aβ such as $A\beta_{13-42}$. $A\beta_{Insoluble}$ refers to forms of Aβ that are recovered as described in Gravina, *J. Biol. Chem.* 270:7013-7016 (1995). APPβ can also be specifically measured to assess the amount of β-secretase activity (Seubert *et al.*, *Nature* 361:260-263 (1993)). Several of these Aβ

forms and their association with Alzheimer's disease are described by Haass and Selkoe (1993). Detection and measurement of $A\beta_{tot}$, $A\beta_{1-42}$, and $A\beta_{X-42}$ are described in Example 6. Generally, specific forms of $A\beta$ can be assayed, either quantitatively or qualitatively using specific antibodies, as described below. When referring to amino acid positions in forms of $A\beta$, the positions correspond to the $A\beta$ region of APP. Amino acid 1 of $A\beta$ corresponds to amino acid 672 of APP, and amino acid 42 of $A\beta$ corresponds to amino acid 714 of APP.

Also preferred as targets for assay measurement are APP markers. For example, different forms of secreted APP (termed APPα and APPβ) can also be measured (Seubert *et al.*, *Nature* 361:260-263 (1993)). Other APP forms can also serve as targets for assays to assess the potential for compounds to affect Alzheimer's disease. These include FLAPP+APPα, full length APP, C-terminal fragments of APP, especially C100 (the last 100 amino acids of APP) and C57 to C60 (the last 57 to 60 amino acids of APP), and any forms of APP that include the region corresponding to $A\beta_{1-40}$.

APP forms are also preferred targets for assays to assess the potential for compounds to affect Alzheimer's disease. The absolute level of APP and APP transcripts, the relative levels of the different APP forms and their cleavage products, and localization of APP expression or processing are all markers associated with Alzheimer's disease that can be used to measure the effect of treatment with potential therapeutic compounds. The localization of APP to plaques and neuritic tissue is an especially preferred target for these assays.

Quantitative measurement can be accomplished using many standard assays. For example, transcript levels can be measured using RT-PCR and hybridization methods including RNase protection, Northern analysis, and R-dot analysis. APP and $A\beta$ levels can be assayed by ELISA, Western analysis, and by comparison of immunohistochemically stained tissue sections. Immunohistochemical staining can also be used to assay localization of APP and $A\beta$ to particular tissues and cell types. Such assays were described above and specific examples are provided below.

B. Plaque-related Markers.

A variety of other molecules are also present in plaques of individuals with AD and in the disclosed transgenic animals, and their presence in plaques and neuritic tissue can be detected. The amount of these markers present in plaques or neuritic tissue is expected to increase with the age of untreated transgenic animals. Preferred plaque-related markers are apolipoprotein E, glycosylation end products, amyloid P component, advanced glycosylation end products (Smith *et al.*, *Proc. Natl. Acad. Sci. USA* 91:5710 (1994)), growth inhibitory factor, laminin, collagen type IV (Kalaria and Perry (1993); Ueda *et al.* (1993)), receptor for advanced glycosylation products (RAGE), and ubiquitin.

While the above markers can be used to detect specific components of plaques and neuritic tissue, the location and extent of plaques can also be determined by using well known histochemical stains, such as Congo Red and thioflavin S, as described above and in some examples below.

C. Cytoskeletal and Neuritic Markers.

Many changes in cytoskeletal markers associated with AD have also been detected in transgenic PDAPP mice. These markers can be used in AD screening assays to determine the effect of compounds on AD. Many of the changes in cytoskeletal markers occur either in the neurofibrillary tangles or dystrophic neurites associated with plaques (Kosik *et al.* (1992); Lovestone and Anderton (1992); Brandan and Inestrosa (1993); Trojanowski *et al.* (1993); Masliah *et al.* (1993)).

The following are preferred cytoskeletal and neuritic markers that exhibit changes in and/or an association with AD. These markers can be detected, and changes can be determined, to measure the effect of compounds on the disclosed transgenic animals. Spectrin exhibits increased breakdown in AD. Tau and neurofilaments display an increase in hyperphosphorylation in AD, and levels of ubiquitin increase in AD. Tau, ubiquitin, MAP-2, neurofilaments, heparin sulfate, and chrondroitin sulphate are localized to plaques and neuritic tissue in AD and in general change from the normal localization. GAP43 levels are decreased in the hippocampus and abnormally

phosphorylated tau and neurofilaments are present in PDAPP transgenic mice.

D. Inflammatory Markers.

Alzheimer's disease is also known to stimulate an immunoinflammatory response, with a corresponding increase in inflammatory markers (Frederickson and Brunden (1994); McGeer *et al.* (1991); Wood *et al.* (1993)). The following are preferred inflammatory markers that exhibit changes in and/or an association with AD. Detection of changes in these markers are useful in AD screening assays. Acute phase proteins and glial markers, such as α1-antitrypsin, C-reactive protein, α2-macroglobulin (Tooyama *et al.*, *Molecular & Chemical Neuropathology* 18:153-60 (1993)), glial fibrillary acidic protein (GFAP), Mac-1, F4/80, and cytokines, such as IL-1α and β, TNFα, IL-8, MIP-1α (Kim *et al.*, *J. Neuroimmunology* 56:127-134 (1995)), MCP-1 (Kim *et al.*, *J. Neurological Sciences* 128:28-35 (1995); Kim *et al.*, *J. Neuroimmunology* 56:127-134 (1995); Wang *et al.*, *Stroke* 26:661-665 (1995)), and IL-6, all increase in AD and are expected to increase in the disclosed transgenic animals. Complement markers, such as C3d, C1q, C5, C4d, C4bp, and C5a-C9, are localized in plaques and neuritic tissue. Major histocompatibility complex (MHC) glycoproteins, such as HLA-DR and HLA-A, D,C increase in AD. Microglial markers, such as CR3 receptor, MHC I, MHC II, CD 31, CD11a, CD11b, CD11c, CD68, CD45RO, CD45RD, CD18, CD59, CR4, CD45, CD64, and CD44 (Akiyama *et al.*, *Brain Research* 632:249-259 (1993)) increase in AD. Additional inflammatory markers useful in AD screening assays include α2 macroglobulin receptor, Fibroblast growth factor (Tooyama *et al.*, *Neuroscience Letters* 121:155-158 (1991)), ICAM-1 (Akiyama *et al.*, *Acta Neuropathologica* 85:628-634 (1993)), Lactotransferrin (Kawamata *et al.*, *American Journal of Pathology* 142:1574-85 (1993)), C1q, C3d, C4d, C5b-9, Fc gamma RI, Fc gamma RII, CD8 (McGeer *et al.*, *Can J Neurol Sci* 16:516-527 (1989)), LCA (CD45) (McGeer *et al.* (1989); Akiyama *et al.*, *Journal of Neuroimmunology* 50:195-201 (1994)), CD18 (beta-2 integrin) (Akiyama and McGeer, *Journal of Neuroimmunology* 30:81-93 (1990)),

CD59 (McGeer *et al.*, *Brain Research* 544:315-319 (1991)), Vitronectic (McGeer *et al.*, *Canadian Journal of Neurological Sciences* 18:376-379 (1991); Akiyama *et al.*, *Journal of Neuroimmunology* 32:19-28 (1991)), Vitronectin receptor, Beta-3 integrin (Akiyama *et al.* (1991)), Apo J, clusterin (McGeer *et al.*, *Brain Research* 579:337-341 (1992)), type 2 plasminogen activator inhibitor (Akiyama *et al.*, *Neuroscience Letters* 164:233-235 (1993)), CD44 (Akiyama *et al.*, *Brain Research* 632:249-259 (1993)), Midkine (Yasuhara *et al.*, *Biochemical & Biophysical Research Communications* 192:246-251 (1993)), Macrophage colony stimulating factor receptor (Akiyama *et al.*, *Brain Research* 639:171-174 (1994)), MRP14, 27E10, and interferon-alpha (Akiyama *et al.*, *Journal of Neuroimmunology* 50:195-201 (1994)). Additional markers which are associated with inflammation or oxidative stress include 4-hydroxynonenal-protein conjugates (Uchida *et al.*, *Biochem. Biophys. Res. Comm.* 212:1068-1073 (1995); Uchida and Stadtman, *Methods in Enzymology* 233:371-380 (1994); Yoritaka *et al.*, *Proc. Natl. Acad. Sci. USA* 93:2696-2701 (1996)), IκB, NFκB (Kaltschmidt *et al.*, *Molecular Aspects of Medicine* 14:171-190 (1993)), $cPLA_2$ (Stephenson *et al.*, *Neurobiology Dis.* 3:51-63 (1996)), COX-2 (Chen *et al.*, *Neuroreport* 6:245-248 (1995)), Matrix metalloproteinases (Backstrom *et al.*, *J. Neurochemistry* 58:983-992 (1992); Bignami *et al.*, *Acta Neuropathologica* 87:308-312 (1994); Deb and Gottschall, J. *Neurochemistry* 66:1641-1647 (1995); Peress *et al.*, *J. Neuropathology & Experimental Neurology* 54:16-22 (1995)), Membrane lipid peroxidation, Protein oxidation (Hensley *et al.*, *J. Neurochemistry* 65:2146-2156 (1995); Smith *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10540-10543 (1991)), and diminished ATPase activity (Mark *et al.*, *J. Neuroscience* 15:6239 (1995)). These markers can be detected, and changes can be determined, to measure the effect of compounds on the disclosed transgenic animals.

E. Neuronal and Neurotransmitter-related Markers.

Changes in neuronal and neurotransmitter biochemistry have been associated with AD and in the disclosed PDAPP animals. In AD there is a profound reduction in cortical and hippocampal cholinergic innervation. This

is evidenced by the dramatic loss of the synthetic enzyme choline acetyltransferase and decreased acetylcholinersterase, synaptosomal choline uptake (as measured by hemicholinium binding) and synthesis and release of acetylcholine (Rylett *et al.* (1983); Sims *et al.* (1980); Coyle et al., *Science* 219:1184-1190 (1983); Davies and Maloney, *Lancet* 2:1403 (1976); Perry *et al.*, *Lancet* 1:189 (1977); Sims *et al.*, *J. Neurochem.* 40: 503-509 (1983)) all of which are useful markers. These markers can be used in AD screening assays to determine the effect of compounds on AD. There is also a loss of basal forebrain neurons and the galanin system becomes hypertrophic in AD.

In addition to changes in the markers described above in AD, there is also atrophy and loss of basal forebrain cholinergic neurons that project to the cortex and hippocampus (Whitehouse *et al.*, *Science* 215:1237-1239 (1982)), as well as alterations of entorhinal cortex neurons (Van Hoesan *et al.*, *Hippocampus* 1:1-8 (1991). Based upon these observations measurement of these enzyme activities, neuronal size, and neuronal count numbers are expected to decrease in the disclosed transgenic animals and are therefore useful targets for detection in AD screening assays. Basal forebrain neurons are dependent on nerve growth factor (NGF). Brain-derived neurotrophic factor (BDNF) may also decrease in the hippocampus in the disclosed transgenic animals and is therefore a useful target for detection in AD screening assays.

It has also been shown that APP and Aβ release are affected by stimulation of muscarinic receptors both *in vitro* in tissue culture as well as in brain slices. Similar findings have also been obtained with application of other agonists linked to phosphoinosital turnover (Nitsch *et al.* (1992); Hung *et al.*, *J. Biol. Chem.* 268:22959-22962 (1993); Nitsch *et al.*, *Proceedings of the Eighth Meeting of the International Study Group on the Pharmacology of Memory Disorders Associated with Aging* 497-503 (1995); Masliah and Terry (1993); Greenamyre and Maragos (1993); McDonald and Nemeroff (1991); Mohr *et al.* (1994); Perry, *British Medical Bulletin* 42:63-69 (1986); Masliah *et al.*, *Brian Research* 574:312-316 (1992); Schwagerl *et al.*, *Journal of Neurochemistry* 64:443-446 (1995)). Based upon these observations, it is

possible that neurotransmitter agonists will reduce the production of Aβ in the disclosed transgenic animals. Based on this reasoning, screening assays that measure the effect of compounds on neurotransmitter receptors can possibly be used to identifying compounds useful in treating AD.

In addition to the well-documented changes in the cholinergic system, dysfunction in other receptor systems such as the serotinergic, adrenergic, adenosine, and nicotine receptor systems, has also been documented. Markers characteristic of these changes, as well as other neuronal markers that exhibit both metabolic and structural changes in AD are listed below. Changes in the level and/or localization of these markers can be measured using similar techniques as those described for measuring and detecting the earlier markers.

The following are preferred cytoskeletal and neuritic markers that exhibit changes in and/or an association with AD. Levels of cathepsin (cat) D,B and Neuronal Thread Protein, and phosphorylation of elongation factor-2, increase in AD. Cat D,B, protein kinase C, and NADPH are localized in plaque and neuritic tissue in AD. Activity and/or levels of nicotine receptors, 5-HT_2 receptor, NMDA receptor, α2-adrenergic receptor, synaptophysin, p65, glutamine synthetase, glucose transporter, PPI kinase, drebrin, GAP43, cytochrome oxidase, heme oxygenase, calbindin, adenosine A1 receptors, mono amine metabolites, choline acetyltransferase, acetylcholinesterase, and symptosomal choline uptake are all reduced in AD.

Additional markers that are associated with AD or after treatment of cells with Aβ include (1) $cPLA_2$ (Stephenson *et al.*, *Neurobiology of Diseases* 3:51-63 (1996)), which is upregulated in AD, (2) Heme oxygenase-1 (Premkumar *et al.*, *J. Neurochemistry* 65:1399-1402 (1995); Schipper *et al.*, *Annals of Neurology* 37:758-768 (1995); Smith *et al.*, *American Journal of Pathology* 145:42-47 (1994); Smith *et al.*, *Molecular & Chemical Neuropathology* 24:227-230 (1995)), c-jun (Anderson *et al.*, *Experimental Neurology* 125:286-295 (1994); Anderson *et al.*, *J. Neurochemistry* 65:1487-1498 (1995)), c-fos (Anderson *et al.* (1994); Zhang *et al.*, *Neuroscience* 46:9-21 (1992)), HSP27 (Renkawek *et al.*, *Acta Neuropathologica* 87:511-

519 (1994); Renkawek *et al.*, *Neuroreport* 5:14-16 (1993)), HSP70 (Cisse *et al.*, *Acta Neuropathologica* 85:233-240 (1993)), and MAP5 (Geddes *et al.*, *J. Neuroscience Research* 30:183-191 (1991); Takahashi *et al.*, *Acta Neuropathologica* 81:626-631 (1991)), which are induced in AD and in cortical cells after Aβ treatment, and (3) junB, junD, fosB, fra1 (Estus *et al.*, *J. Cell Biology* 127:1717-1727 (1994)), cyclin D1 (Freeman *et al.*, *Neuron* 12:343-355 (1994); Kranenburg *et al.*, *EMBO Journal* 15:46-54 (1996)), p53 (Chopp, *Current Opinion in Neurology & Neurosurgery* 6:6-10 (1993); Sakhi *et al.*, *Proc. Natl. Acad. Sci. USA* 91:7525-7529 (1994); Wood and Youle, *J. Neuroscience* 15:5851-5857 (1995)), NGFI-A (Vaccarino *et al.*, *Molecular Brain Research* 12:233-241 (1992)), and NGFI-B, which are induced in cortical cells after Aβ treatment.

F. Measuring the Amounts and Localization of AD Markers.

Quantitative measurement can be accomplished using many standard assays. For example, transcript levels can be measured using RT-PCR and hybridization methods including RNase protection, Northern analysis, and R-dot analysis. Protein marker levels can be assayed by ELISA, Western analysis, and by comparison of immunohistochemically stained tissue sections. Immunohistochemical staining can also be used to assay localization of protein markers to particular tissues and cell types. The localization and the histopathological association of AD markers can be determined by histochemical detection methods such as antibody staining, laser scanning confocal imaging, and immunoelectron micrography. Examples of such techniques are described in Masliah *et al.* (1993) and in Example 6 below.

In the case of receptors and enzymatic markers, activity of the receptors or enzymes can be measured. For example, the activity of neurotransmitter metabolizing enzymes such as choline acetyltransferase and acetylcholine esterase can be measured using standard radiometric enzyme activity assays.

The activity of certain neurotransmitter receptors can be determined by measuring phosphoinositol (PI) turnover. This involves measuring the

accumulation of inositol after stimulation of the receptor with an agonist. Useful agonists include carbachol for cholinergic receptors and norepinephrine for glutaminergic receptors. The number of receptors present in brain tissue can be assessed by quantitatively measuring ligand binding to the receptors.

The levels and turnover of receptor ligands and neurotransmitters can be determined by quantitative assays taken at various time points. Dopamine turnover can be measured using DOPAC and HVA. MOPEG sulfate can be used to measure norepinephrine turnover and 5-HIAA can be used to measure serotonin turnover. For example, norepinephrine levels have been shown to be reduced 20% in the hippocampus of 12 to 13 month old PDAPP transgenic mice relative to controls. Generally, the above assays can be performed as described in the literature, for example, in Rylett *et al.* (1983); Sims *et al.* (1980); Coyle et al., *Science* 219:1184-1190 (1983); Davies and Maloney, *Lancet* 2:1403 (1976); Perry *et al.*, *Lancet* 1:189 (1977); Sims *et al.*, *J. Neurochem.* 40: 503-509 (1983). These markers are also described by Bymaster *et al.*, *J. Pharm. Exp. Ther.* 269:282-289 (1994).

G. Screening Assays Using Cultured Cells.

Screening assays for determining the therapeutic potential of compounds can also be performed using cells derived from animals transgenic for the disclosed APP constructs and cell cultures stably transfected with the disclosed constructs. For example, such assays can be performed on cultured cells in the following manner. Cell cultures can be transfected generally in the manner described in International Patent Application No. 94/10569 and Citron *et al.* (1995). Derived transgenic cells or transfected cell cultures can then be plated in Corning 96-well plates at 1.5 to 2.5 x 10^4 cells per well in Dulbecco's minimal essential media plus 10% fetal bovine serum.

Following overnight incubation at 37°C in an incubator equilibrated with 10% carbon dioxide, media are removed and replaced with media containing a compound to be tested for a two hour pretreatment period and cells were incubated as above. Stocks containing the compound to be tested

are first prepared in 100% dimethylsulfoxide such that at the final concentration of compound used in the treatment, the concentration of dimethylsulfoxide does not exceed 0.5%, preferably about 0.1%.

At the end of the pretreatment period, the media are again removed and replaced with fresh media containing the compound to be tested as above and cells are incubated for an additional 2 to 16 hours. After treatment, plates are centrifuged in a Beckman GPR at 1200 rpm for five minutes at room temperature to pellet cellular debris from the conditioned media. From each well, 100 μL of conditioned media or appropriate dilutions thereof are transferred into an ELISA plate precoated with antibody 266 (an antibody directed against amino acids 13 to 28 of Aβ) as described in International Patent Application No. 94/10569 and stored at 4°C overnight. An ELISA assay employing labelled antibody 6C6 (against amino acids 1 to 16 of Aβ) can be run to measure the amount of Aβ produced. Different capture and detection antibodies can also be used.

Cytotoxic effects of the compounds are measured by a modification of the method of Hansen *et al.*, *J. Immun. Method.* 119:203-210 (1989). To the cells remaining in the tissue culture plate, 25 μL of a 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/mL) is added to a final concentration of 1 mg/mL. Cells are incubated at 37°C for one hour, and cellular activity is stopped by the addition of an equal volume of MTT lysis buffer (20% w/v sodium dodecylsulfate in 50% dimethylformamide, pH 4.7). Complete extraction is achieved by overnight shaking at room temperature. The difference in the OD_{562nm} and the OD_{650nm} is measured in a Molecular Device's UV_{max} microplate reader, or equivalent, as an indicator of the cellular viability.

The results of the Aβ ELISA are fit to a standard curve and expressed as ng/mL Aβ. In order to normalize for cytotoxicity, these results are divided by the MTT results and expressed as a percentage of the results from a control assay run without the compound.

All publications cited herein are hereby incorporated by reference. The information contained in these publications for which the publications are cited is generally known.

Example 1: Expression of pMTAPP-1 in NIH3T3 and PC12 Cells.

The clone pMTAPP-1 is an example of an APP770 expression construct as shown in Figure 1a where the promoter used is the metallothionine promoter. Stable cell lines were derived by transfecting NIH3T3 and PC12 cell lines (ATCC #CCL92 and CRL1721). Five hundred thousand NIH3T3 or PC12 cells were plated into 100 mm dishes and transfected with a mixture of 5 mg of the *SalI* fragment and 1 mg of pSV2neo DNA (Southern and Berg (1982)) precipitated in the presence of 50 mg lipofectin (Gibco, BRL) in a final volume of 100 μl. Polylysine-coated plates were used for PC12 cells, which normally do not adhere well to tissue culture dishes. The cells were fed with selection medium containing 10% fetal bovine serum in DMEM or RPMI and supplemented with G418. Five hundred mg/ml (biological weight) and 250 mg/ml of G418 were used to select colonies form NIH3T3 and PC12 cells, respectively. Fifteen days after transfection, colonies of cells resistant to G418 were isolated by cloning rings and expanded in T flasks. The presence of APP cDNA in the cells was detected by PCR using the procedure of Mullis and Faloona, *Methods Enzymol.* 155:335-350 (1987), the teachings of which are generally known and are incorporated herein.

Expression of APP in 25 colonies from each cell line was analyzed by immunostaining (Majocha *et al.* (1988)). Cells were grown to subconfluence and fixed in a solution containing 4% paraformaldehyde, 0.12 M NaCl, and 20 mm Na_3PO_4, pH 7.0. They were incubated overnight with a primary monoclonal antibody against a synthetic Aβ sequence (Masters *et al.* (1985); Glenner and Wong) provided by Dr. Ronald Majocha, Massachusetts General Hospital, Boston, MA, followed by a generalized anti-mouse antibody conjugated to biotin (Jackson ImmunoResearch Labs, PA). Immunostaining was then performed by adding avidin-horseradish peroxidase (HRP) (Vector Labs, Burlingame, CA) and diaminobenzidine as the chromogen (Majocha *et*

al. (1985)). The results indicated that the pMTAPP-1 vector was expressing APP in both NIH3T3 and PC12 cells.

Example 2: Expression of pEAPP-1 in PC12 Cells.

pEAPP-1 is an example of an APP770 expression construct as shown in Figure 1a where the promoter used is the 25 kb human APP gene promoter. DNA from this construct was transfected into PC12 cells as described above. Certain clones of pEAPP-1 transfected cells exhibited a differentiation phenotype morphologically similar to that exhibited by PC12 cells treated with nerve growth factor (NGF). PC12 cells normally are fairly round and flat cells. Those transfected with pEAPP-1 have cytoplasmic extensions resembling neurites. PC12 cells treated with NGF extend very long neuritic extensions. Thirteen PC12 cell clones transfected with pEAPP-1 were selected and propagated. Eight of these cell clones exhibited the spontaneous differentiation phenotype with clones 1-8, 1-1, and 1-4 exhibiting the strongest phenotypes. Staining of pEAPP-1 transfected PC12 cells with antibody against the Aβ as described in Example 1 indicated that those cells exhibiting the differentiation were also expressing APP. Because PC12 cells transfected with the pMTAPP-1 clone did not exhibit this phenotype even though the APP770 cDNA is expressed, these results suggest that expression of APP770 from the human promoter has novel properties regarding the physiology of the cell.

Example 3: Expression of pMTA4 in PC12 Cells.

pMTA4 is an example of the type of construct shown in Figure 4a where the promoter used is the metallothionine promoter. The protein encoded by this construct differs slightly from that depicted in Figure 4a. An APP770 cDNA clone was digested with *Asp*718 which cleaves after position 57 (number system of Kang *et al.* (1987)). The resulting 5' extension was filled in using the Klenow enzyme (Sambrook *et al.* (1989)). The same DNA preparation was also cleaved with *Eco*RI which also cuts after position 2020 and the resulting 5' extension was filled in using the Klenow enzyme (Sambrook *et al.* (1989)). Self-ligation of this molecule results in an expression clone in which the truncated protein thus encoded contains the

leader sequence, followed by a shortened version of the Aβ starting with the sequence Phe-Arg-Val-Gly-Ser-of the Aβ followed by the 56 terminal amino acids of APP. DNA from this construct was transfected into PC12 cells as described above.

Example 4: Generation of Transgenic Mice expressing APP under the control of the MT-1 promoter.

Transgenic mice were made by microinjecting pMTAPP-1 vector DNA into pronuclear embryos. pMTAPP-1 is an example of the type of construct shown in Figure 1a in which the APP770 coding sequence is operably linked to the metallothionine promoter. The procedures for microinjection into mouse embryos are described in *Manipulating the Mouse Embryo* by Hogan *et al.* (1986). Only a brief description of the procedures is described below.

Mice were obtained from Taconic Laboratories (German Town, New York). Swiss Webster female mice were used for embryo retrieval and implantation. B6D2F_1 males were used for mating and vasectomized Swiss webster studs were used to simulate pseudopregnancy.

A. Embryo Recovery.

Female mice, 4 to 8 weeks of age, were induced to superovulate with 5 IU of pregnant mare's serum gonadotropin (PMSG; Sigma) followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG; Sigma). Females were placed with males immediately after hCG injection. Embryos were recovered from excised oviducts of mated females 21 hours after hCG in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells were removed with hyaluronidase (1 mg/ml). Pronuclear embryos were then washed and placed in Earle's balanced salt solution containing 0.4% BSA (EBSS) in a 37.5°C incubator with a humidified atmosphere at 7% CO_2, 5% O_2, and 88% N_2 until the time of injection.

B. Microinjection.

Elutip-D™ purified *SalI* DNA was dissolved in 5 mM Tris (pH 7.4) and 0.1 mM EDTA at 3 μg/ml concentration for microinjection.

Microneedles and holding pipettes were pulled from Fisher coagulation tubes (Fisher) on a DKI model 720 pipette puller. Holding pipettes were then broken at approximately 70 μm (O.D.) and fire polished to an I.D. of about 30 μm on a Narishige microforge (model MF-83). Pipettes were mounted on Narishige micromanipulators which were attached to a Nikon Diaphot microscope. The air-filled injection pipette was filled with DNA solution through the tip after breaking the tip against the holding pipette. Embryos, in groups of 30 to 40, were placed in 100 μl drops of EBBS under paraffin oil for micromanipulation. An embryo was oriented and held with the holding pipette. The injection pipette was then inserted into the male pronucleus (usually the larger one). If the pipette did not break through the membrane immediately the stage was tapped to assist in penetration. The nucleus was then injected and the injection was monitored by swelling of the nucleus. Following injection, the group of embryos was placed in EBSS until transfer to recipient females.

C. Transfer.

Randomly cycling adult female mice were paired with vasectomized Swiss Webster males. Recipient females were mated at the same time as donor females. At the time of transfer, the females were anesthetized with avertin. The oviducts were exposed by a single midline dorsal incision. An incision was then made through the body wall directly over the oviduct. The ovarian bursa was then torn with watch makers forceps. Embryos to be transferred were placed in DPBS and in the tip of a transfer pipet (about 10 to 12 embryos). The pipet tip was inserted into the infundibulum and embryos were transferred. After the transfer, the incision was closed by two sutures.

D. Analysis Of Mice For Transgene Integration.

At three weeks of age or older, tail samples about 1 cm long were excised for DNA analysis. The tail samples were digested by incubating with shaking overnight at 55°C in the presence of 0.7 ml 5 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS and 350 μg of proteinase K. The digested material was extracted once with an equal volume of phenol and once with an equal

volume of phenol:chloroform (1:1 mixture). The supernatants were mixed with 70 μl 3 M sodium acetate (pH 6.0) and the DNA was precipitated by adding equal volume of 100% ethanol. The DNA was spun down in a microfuge, washed once with 70% ethanol, dried and dissolved in 100 μl TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA).

Ten to twenty microliters of DNA from each sample was digested with *Bam*HI, electrophoresed on 1% agarose gels, blotted onto nitrocellulose paper, and hybridized with ^{32}P-labeled APP cDNA fragment. Transgenic animals were identified by autoradiography of the hybridized nitrocellulose filters. The DNAs were also analyzed by PCR carried out by synthetic primers to generate an 800 bp fragment of APP DNA.

A total of 671 pronuclear embryos were microinjected out of which 73 live and 6 dead pups were born. DNA analysis identified 9 transgenic mice (5 females and 4 males) which were bred to generate F_1 and F_2 transgenics. These animals can be analyzed for expression of mRNA and protein of APP in different tissues and for analysis of behavioral and pathological abnormalities as described above. Transgenic mice with this construct express transgenic RNA.

Example 5: Construction of APP construct containing a combination cDNA/genomic coding sequence.

A cDNA/genomic APP construct containing introns 6, 7 and 8 was prepared by combining APP cDNA encoding exons 1-6 and 9-18 with genomic APP sequences encoding introns 6, 7 and 8, and exons 7 and 8 (see Figure 6). In order to create a splicing cassette small enough for convenient insertion in a pUC vector, two deletions in intronic sequences were made. A deletion was made in intron 6 from position 143 of intron 6 to the *Bam*HI site located upstream of the beginning of exon 7 (1658 bp before the beginning of exon 7). Another deletion was made in intron 8 from the first *Bam*HI site in intron 8 to a site at 263 bp before the beginning of exon 9. To avoid confusion, these truncated forms of APP introns 6 and 8 are referred to herein as intron Δ6 and intron Δ8. *Bam*HI sites were engineered at the sites of these deletions, so that they are marked by the presence of

*Bam*HI sites. In this construct, referred to as PDAPP, exons 7 and 8 and intron 7 are intact genomic sequences, except that the unique *Xho*I site in intron 7 was destroyed.

DNA fragments containing the truncated introns were generated as follows: a *Bam*HI site was engineered 143 bp into intron 6 nucleotide by PCR mutagenesis ("Mutagenesis by PCR" in *PCR Technology: Current Innovations* (Griffith and Griffith, eds., CRC Press, 1994) pages 69-83) and another *Bam*HI site was engineered by PCR mutagenesis 263 bp prior to the beginning of exon 9. These sites were engineered into separate APP genomic DNA clones containing the junctions of exon 6 and intron 6, and intron 8 and exon 9, respectively, resulting in modified APP genomic DNA clones.

The entire cassette was assembled in the APP cDNA clone as follows (Figure 11). The 889 bp *Bam*HI to *Xcm*I fragment of APP cDNA containing exons 1 through 5 and part of exon 6 (including nucleotides 1 to 843 of SEQ ID NO:5) was cloned into a vector containing *Bam*HI and *Xho*I sites downstream from the insertion site to make APP770x-oligo-x. APP770x-oligo-x was then cut with *Xcm*I and *Bam*HI. Then two fragments were obtained from the modified APP genomic DNA clone containing the junction of exon 6 and intron 6 described above by cutting with *Xcm*I and *Bam*HI. The resulting 34 bp fragment from the *Xcm*I in exon 6 to the *Xcm*I in intron 6, and 131 bp fragment from the *Xcm*I in intron 6 to the artificially created *Bam*HI site at position 143 bp of intron 6 were ligated into APP770x-oligo-x in a three-way ligation step to make APP770x-E6oligo-x. The orientation of the fragments was confirmed by sequencing. APP770x-E6oligo-x was then cut with *Bam*HI and *Xho*I. Then the 313 bp *Bam*HI and *Xho*I fragment from the modified APP genomic DNA clone containing the junction of intron 8 and exon 9 was ligated into APP770x-E6oligo-x to make APP770xE6E9x.

APP770xE6E9x was then cut with *Bam*HI and the 6.8 kb *Bam*HI fragment of APP genomic DNA encoding the KPI and OX-2 domains (exons 7 and 8) was inserted at this site. This fragment starts at the *Bam*HI site 1658 bp upstream of the start of exon 7 and extends to the first *Bam*HI site in intron 8. This *Bam*HI fragment was obtained from a lambda phage genomic

clone encoding this portion of the APP gene, that was obtained from a Human Placental genomic library in the Lambda FIXII vector obtained from Stratagene. This *Bam*HI fragment originally contained an *Xho*I site which was destroyed by cutting, filling in, and religation. The locations of the deletions are diagramed in Figure 10. This clone, containing exons 1-8 and part of 9, and introns 6, 7 and 8, was termed the "APP splicing cassette." The APP splicing cassette was cut out with *Nru*I and *Xho*I and used to replace the *Nru*I to *Xho*I cDNA fragment of APP cDNA bearing the Hardy mutation. This mutant form of APP cDNA was produced by converting the G at nucleotide position 2145 to T by site directed mutagenesis. This changes the encoded amino acid from Val to Phe. The resulting construct is a combination cDNA/genomic APP "minigene."

Sequencing of the 6.8 kb *Bam*HI fragment containing APP exons 7 and 8 derived from the APP genomic clone used to generate this construct showed that intron 7 is 2.6 kb long, and that the first *Bam*HI site in intron 8, the upstream site of the deletion in intron 8 engineered into the APP minigene construct, is 2329 bp downstream from the end of exon 8. This does not coincide with the restriction map of the APP gene published by Yoshikai *et al.* (1990) and Yoshikai *et al.* (1991). Comparison of their map to our sequence indicates that Yoshikai *et al.* switched the order of two *Eco*RI fragments in their restriction mapping. The 1.60 kb *Eco*RI fragment containing exon 8 is actually upstream of the 1.48 kb *Eco*RI fragment and the 1.48 kb *Eco*RI fragment Yoshikai *et al.* mapped in intron 7 is actually in intron 8. We have confirmed this location for the *Eco*RI fragment containing exon 8 by sizing of PCR generated fragments from human DNA.

This APP minigene was operatively linked to the PDGF-B promoter to provide expression of the APP cDNA/genomic construct in mammalian cells. The PDGF β-chain 5' flanking sequence was inserted upstream of the NruI site at the beginning of the APP minigene. This fragment includes 1.3 kb upstream of the transcription initiation site, where the PDGF-B promoter resides, and approximately 70 bp of 5' untranslated region, ending at the A*ur*II site (Higgins *et al.* (1994)). The late SV40 polyadenylation signal,

carried on a 240 bp B*am*HI to B*cl*I fragment, was added downstream of the APP minigene. This construct, combining the PDGF-B promoter, the APP splicing cassette, the Hardy mutation, and the SV40 polyadenylation signal is referred to as PDAPP (Figure 9).

Example 6: Transgenic mice containing the PDAPP construct.

Transgenic mice were generated using the PDAPP construct described in Example 5. Transgenic mice were generated by microinjection using standard techniques as described above. PDAPP DNA was microinjected into the embryos at the two-cell stage. Plasmid sequences (pUC) were removed by *Sac*I and *Not*I digestion before microinjection. Seven founder mice were generated and line 109 was used for extensive analysis. Only heterozygous animals were used. Southern analysis of 104 animals from four generations showed that approximately 40 copies of the transgene were inserted at a single site and transmitted in a stable manner. Human APP messenger RNA was produced in several tissues of the transgenic mouse, but at especially high levels in brain. RNase protection assays revealed at least 20-fold more APP expression in the brains of line 109 animals than in the mouse lines expressing neuron-specific enolase (NSE)-promoter-driven APP transgenes previously described by Quon *et al.* (1991), Mucke *et al.*, *Brain Res.* 666:151-167 (1994), McConlogue *et al.*, *Neurobiol. Aging* 15:S12 (1994), and Higgins *et al.*, *Ann Neurol.* 35:598-607 (1994).

A. Expression of APP Transcripts and Protein.

RNA was isolated from brain tissue as described by Chomaczynski and Sacchi, *Analyt. Biochem.* 162:156-159 (1987), and subjected to RT-PCR as described by Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:9717-9721 (1989), using human-specific APP primers (5'-CCGATGATGACGAGGACGAT-3', SEQ ID NO:7; 5'-TGAACACGTGACGAGGCCGA-3', SEQ ID NO:8) using 40 cycles of 1 minute at 94°C, 40 seconds at 60°C, and 50 seconds at 72°C. RT-PCR analysis demonstrated the presence of transcripts encoding the 695, 751 and 770 isoforms of human APP in transgenic animal brains but not in brains from non-transgenic littermates. The identities of the human APP RT-PCR

bands from the transgenic mouse RNA were verified by subcloning and sequencing.

The relative levels and alternative splicing of APP transcripts in brains of PDAPP transgenic mice, NSE-APP transgenic mice, non-transgenic mice, and humans with and without AD were compared in RNase protection assays (Rockenstein *et al.*, *J. Biol. Chem.* 270:28257-28267 (1995)). PDAPP mice expressed approximately 5-fold higher total APP mRNA levels than non-transgenic controls, and at least 20-fold higher human APP mRNA levels than most NSE-APP transgenic mice. While NSE-driven human APP expression does not affect the levels of murine APP mRNA, PDAPP transgenic mice showed a significant 30% decrease in murine APP transcripts. While the relative abundances of murine APP770:751:695 mRNAs in non-transgenic mouse brains were roughly 1:1:35, the corresponding human APP mRNA levels in PDAPP transgenic mouse brains were 5:5:1.

Analysis of holo-APP was performed by brain homogenization in 10 volumes of PBS containing 0.5 mM EDTA, 10 $\mu g\ ml^{-1}$ leupeptin and 1 mM PMSF. Samples were spun at 12,000*g* for 10 min and the pellets resuspended in RIPA (150 mM NaCl, 50 mM Tris, ph 8.0, 20 mM EDTA, 1.0% deoxycholate, 1.0% Triton X-100, 0.1% SDS, 1 mM PMSF and 10 $\mu g\ ml^{-1}$ leupeptin). Samples (each containing 30 μg total protein) were analyzed by SDS-PAGE, transferred to Immobilon membranes and reacted with either the holo-APP antibody, anti-6 (anti Bx 6), described by Oltersdorf *et al.*, *J. Biol. Chem.* 285:4492-4497 (1990), or 8E5 monoclonal antibody. 8E5 was prepared against a bacterial fusion protein encompassing human APP residues 444-692 (Oltersdorf *et al.* (1990)) and is human-specific, showing essentially no crossreactivity against mouse APP. Immunoblot analysis of total APP expression (human and mouse) in transgenic mouse line 109 and control littermate brain tissue using C-terminal APP antibody anti-6 showed much higher levels of expression in the transgenic mice. Immunoblot analysis of brain homogenates using either the holo-APP polyclonal antibody anti-6 or the human-specific APP monoclonal antibody 8E5 revealed human APP over-

expression in the transgenic mouse at levels at least 3-fold higher in hippocampus than either endogenous mouse APP levels or those in AD brain.

For immunoblot analysis of Aβ, a 9-month-old mouse brain was homogenized in 5 ml 6 M guanidine HCl, 50 mM Tris, pH 7.5. The homogenate was centrifuged at 100,000*g* for 15 min and the supernatant was dialyzed against H_20 overnight adjusted to PBS with 1 mM PMSF and 25 μg ml^{-1} leupeptin. This material was immunoprecipitated with antibody 266 resin, and immunoblotted with the human-specific Aβ antibody, 6C6, as described by Seubert *et al.*, *Nature* 359:325-327 (1992). Using this human-specific Aβ antibody (6C6), a 4 kD β amyloid-immunoreactive peptide was isolated from the brains of the transgenic animals, which corresponds to the relative molecular mass of Aβ. Brain levels of Aβ were at least 10-fold higher in line 109 animals than in the previously described human APP transgenic mice. Embryonic day 16 cortical cell cultures from transgenic animals constitutively secreted human Aβ, including a substantial fraction of Aβ 1-42 (5 ng ml^{-1} total Aβ; 0.7 ng ml^{-1} Aβ 1-42), as detected in media by human-specific Aβ enzyme-linked immunosorbent assays, as described by Seubert *et al.* (1992) and McConlogue *et al.* (1994), and as described in Example 8. Thus, line-109 animals greatly overexpressed human APP mRNA, holo-APP and Aβ in their brains.

B. Histopathology of PDAPP Transgenic Mice.

Brains from 180 transgenic and 160 age-matched non-transgenic age-matched controls (4 to 20 months old) representing five generations of the line 109 pedigree were extensively examined histopathologically. Some mouse brains were removed and placed in alcohol fixative (Arai *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87:2249-2253 (1990)) for 48 hours before paraffin embedding. Other mice were perfused with saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate. For paraffin embedded brains, 6 μm coronal or parasaggital sections from transgenic and non-transgenic mice were placed adjacent to each other on poly-L-lysine coated slides. The sections were deparaffinized, rehydrated and treated with 0.03% H_2O_2 for 30 min before overnight incubation at 4°C with a 1:1,000 dilution

of the Aβ antibody, R1280 (Tamaoka *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:1345-1349 (1992)). For absorption studies, synthetic human Aβ 1-40 peptide (Games *et al.*, *Neurobiol. Aging* 13:569-576 (1992)) in 10% aqueous dimethylsulphoxide was added to a final concentration of 7.0 μM to the diluted antibody and incubated for 2 hours at 37°C. The diluent was applied to the sections and processed under the same conditions as the standard antibody solution. Peroxidase rabbit IgG kit (Vector Labs) was then used as recommended, with 3,3'-diaminobenzidine (DAB) as the chromogen. Similarly fixed human AD brain was processed simultaneously under identical conditions.

Before 4 months of age, no obvious Aβ deposition was detected. However, by approximately 4 months of age, the transgenic animals began to exhibit deposits of human Aβ in the hippocampus, corpus callosum and cerebral cortex. These Aβ plaques increased with age, and by eight months many deposits of 30 to 200 μm were seen. As the animals aged beyond 9 months, the density of the plaques increased until the Aβ-staining pattern resembled that of AD. Vascular amyloid, another feature of AD pathology, developed in older mice. Robust pathology was also seen in another transgenic line generated from the PDAPP vector (line 35).

Aβ deposits of varying morphology were clearly evident as a result of using a variety of Aβ antibodies, including well characterized human-specific Aβ antibodies and antibodies specific for the free amino and carboxy termini of Aβ 1-42. Antibody 9204, described by Saido *et al.*, *J. Biol. Chem.* 289:15253-15257 (1994), is specific to Aβ 1-5 and was used at a concentration of 7.0 μg ml^{-1}. Antibody 277-2, specific for Aβ 1-42, was prepared by immunizing New Zealand white rabbits with the peptide cysteine-aminoheptanoic acid-Aβ 33-42 conjugated to cationized BSA ('Super Carriers'; Pierce) using a standard immunization protocol (500 μg per injection). Specific antibodies were affinity-purified from serum against the immunogen immobilized on agarose beads. Before incubation with antibody 277-2, sections were treated for 1 to 2 min with 80% formic acid. For detection, the antibody was reacted using the peroxidase rabbit IgG kit

(Vector Labs). The product was then visualized using DAB as the chromogen, Some sections were then incubated overnight at 4°C with a 1:500 dilution of polyclonal anti-GFAP (Sigma). The GFAP antibody was reacted using the alkaline phosphatase anti-rabbit IgG kit and alkaline phosphatase substrate kit 1 (Vector Labs; used according to the manufacturer's recommendations). Additional sections were incubated overnight with the F480 antibody (Serotec) used at a 1:40 dilution to visualize microglial cells. The mouse peroxidase kit (Vector Labs) was then used according to the manufacturer's recommendations. Some sections were stained with thioflavin S using standard procedures (Dickson *et al.*, *Acta Neuropath.* 79:486-493 (1990)) and viewed with ultraviolet light through an FITC filter of maximum wavelength 440 nm.

Serial sections demonstrated many plaques were positively stained with both the 9204 and 277-2 antibodies. The forms of the Aβ deposition ranged from diffuse irregular types to compacted plaques with cores. Roughly spherical, and wispy, irregular deposits, were labelled with antibody 9204 specific for the free amino terminus of Aβ. Astrocytic gliosis associated with Aβ deposition was evident after double immunolabelling with antibodies to glial fibrillary acidic protein (GFAP) and human Aβ. A compacted Aβ core and 'halo' was evident in several plaques. Non-transgenic littermates showed none of these neuropathological changes. Immunostaining was fully absorbable with the relevant synthetic peptide, and was apparent using a variety of processing conditions, including fixation with paraformaldehyde and Trojanowski methods. Many plaques were stained with thioflavin S, and some were also stained using the Bielschowsky silver method and were birefringent with Congo Red, indicating the true amyloid nature of these deposits.

The majority of plaques were intimately surrounded by GFAP-positive reactive astrocytes, similar to the gliosis found in AD plaques. The neocortices of the transgenic mice contained diffusely activated microglial cells, as defined by their amoeboid appearance, shortened processes, and staining with Mac-1 antibody. Staining by antibodies recognizing

phosphorylated neurofilaments and phosphorylated tau indicated that aberrant phosphorylation occurred in PDAPP brain that was similar to AD. These phosphorylations are seen in AD and are thought to preclude formation of neurofibrillary tangles. Although paired helical filaments (PHF) have not yet been detected in PDAPP mice, the detection of abnormally phosphorylated neurofilaments and tau are thought to be associated with, and the initial step in, the formation of PHF in AD.

Clear evidence for neuritic pathology was apparent using both conventional and confocal immunomicroscopy. Forty μm thick vibratome sections were incubated overnight at 4°C with R1280 (1:1,000) in combination with polyclonal anti-synaptophysin (1:150; Dako) or 8E5 (7.0 μg $^{-1}$). Some sections were incubated with anti-synaptophysin or monoclonal anti-MAP 2 (1:20, Boehringer-Mannheim), and then reacted with a goat anti-rabbit biotinylated antibody (1:100) followed by a mixture of FITC-conjugated horse anti-mouse IgG (1:75) and avidin D Texas red (1:100) (Vector Labs). The double-immunolabelled sections were viewed on a Zeiss Axiovert 35 microscope with attached laser confocal scanning system MRC 600 (Bio-Rad). The Texas red channel collected images of the R1280 or synaptophysin labelling, and the FITC channel collected synaptophysin, 8E5, or MAP 2 labelling. Optical z-sections 0.5 μm in thickness were collected from each region, similar to the image processing and storage described by Masliah *et al.*, *J. Neuropath. Exp Neurol.* 52:619-632 (1993).

Many Aβ plaques were closely associated with distorted neurites that could be detected with human APP-specific antibodies and with anti-synaptophysin antibodies, suggesting that these neurites were derived in part from axonal sprouts, as observed in the AD brain. The plaques compressed and distorted the surrounding neuropil, also as in the AD brain. Synaptic and dendritic density were also reduced in the molecular layer of the hippocampal dentate gyrus of the transgenic mice. This was evident by reduced immunostaining for the presynaptic marker synaptophysin and the dendritic marker MAP-2 in AD brain (Masliah *et al.*, *Am. J. Path.* 138:235-246 (1991)).

Confirmation of the presence of extracellular Aβ was obtained using immunoelectron microscopy. For immunoelectron microscopy, mice were perfused with saline followed by 2.0% paraformaldehyde and 1.0% glutaraldehyde in cacodylate buffer. Forty μm thick vibratome sections were incubated with the R1280 antibody, and reacted using a peroxidase rabbit IgG kit (Vector Labs). Immunolabelled sections with Aβ deposits were then fixed in 1.0% ammonium tetraoxide and embedded in epon/araldite before viewing ultrathin sections with a Jeol CX100 electron microscope (Masliah *et al.*, *Acta Neuropath.* 81:428-433 (1991)).

Table 3. Ultrastructural Similarities and Differences Between AD and PDAPP Transgenic Plaques.

	Alzheimer's Disease	PDAPP
Amyloid fibrils		
size	9-11 nm	9-11 nm
electron density	moderate	high
pinocytic vesicles	abundant	occasional
Dystrophic neurites		
TYPE I		
dense laminar bodies	abundant	abundant
synaptic vesicles and contacts	yes	yes
neurofilament accumulation	yes	yes
TYPE II		
paired helical filaments	yes	none?
Cells associated with amyloid formation		
microglia	abundant	occasional
neurons	occasional	abundant
neurosecretory granules	abundant	abundant
rough endoplasmic reticulum	abundant	abundant
coated pits	yes	yes

Tables 3 and 4 present a summary of the above results, showing cytological and pathological similarities between AD and PDAPP mice. For every feature examined, with the exception of paired helical filaments, the PDAPP mice exhibited pathology characteristic of AD. These findings show that production of human APP in transgenic (TG) mice is sufficient to cause not only amyloid deposition, but also many of the complex subcellular degenerative changes associated with AD.

Table 4. Pathology in Alzheimer's Disease and the PDAPP Mouse.

	Alzheimer's Disease	PDAPP
Aβ Deposition into Plaques		
Diffuse	+	+
Neuritic	+	+
Vascular	+	+
Brain Region Specificity	+	+
Neuritic Dystrophy	+	+
Synaptic Loss	+	+
Inflammatory Response		
Astrocytosis	+	+
Microgliosis	+	+
Cytoskeletal Alterations		
Phosphorylated Neurofilaments	+	+
Phosphorylated Tau	+	+
PHF/Tangles	+	-(?)

The most notable feature of these transgenic mice is their Alzheimer-like neuropathology, which includes extracellular Aβ deposition, dystrophic neuritic components, gliosis, and loss of synaptic density with regional specificity resembling that of AD. Plaque density increases with age in these transgenic mice, as it does in humans (Selkoe, *Rev. Neurosi.* 17:489-517 (1994)), implying a progressive Aβ deposition that exceeds its clearance, as also proposed for AD (Maggio *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:5462-5466 (1992)). The PDAPP transgenic mice provide strong new evidence for the primacy of APP expression and Aβ deposition in AD neuropathology. Such mice also provide a sufficiently robust AD model in which to test whether compounds that lower Aβ production and/or reduce its neurotoxicity *in vitro* can produce beneficial effects in an animal model prior to advancing such drugs into human trials.

Example 7: Construction APP transgenes expressing APP from the PDGF-B promoter.

PDAPP transgenic mice contain a splicing cassette that permits expression of all three major human APP isoforms, where expression is driven by the PDGF-B promoter, and which includes a mutation in amino acid 717, the site of familial AD mutations. It is expected that these features, and others described above, can be used independently to produce transgenic mice useful as models of Alzheimer's disease. Some specific examples of such constructs are described below.

A. Construction of PDAPP-wt.

A wild type version of the cDNA/genomic clone PDAPP was constructed in which the mutation to amino acid 717 was replaced with the wild type. This was accomplished by replacing the 1448 bp *Xho*I to *Spe*I fragment of PDAPP, which includes the part of the APP cDNA sequence that encodes the Hardy mutation in which Val717 is replaced by Phe, with the 1448 bp *Xho*I to *Spe*I fragment of a wild type APP clone. This fragment corresponds to the region from position 1135 to 2588 of SEQ ID NO:5. None of the intron sequences of PDAPP are replaced or removed by this substitution. This construct is referred to as PDAPP-wt. A schematic of PDAPP-wt and its construction is shown in Figure 12.

B. Construction of PDAPP-SwHa.

Another version of the cDNA/genomic clone PDAPP was constructed in which the Swedish mutant at amino acids 670 and 671 was introduced. Plasmid pNSE751.delta3'spl.sw contains cDNA of the human APP751 which includes the Swedish mutation of Lys to Asn and Met to Leu at amino acids 670 and 671, respectively. A 563 bp *Eco*RI to *Spe*I fragment from this plasmid was replaced with the corresponding 563 bp *Eco*RI to *Spe*I fragment of PDAPP, which includes an identical part of the APP cDNA sequence with the exception of Phe717 of the Hardy mutation. This fragment corresponds to the region from position 2020 to 2588 of SEQ ID NO:5. This results in pNSE.delta3'spl.sw/ha, which contains both the Swedish mutation at amino acids 670 and 671, and the Hardy mutation at amino acid 717.

The 1448 bp *Xho*I to *Spe*I fragment of PDAPP was then replaced with the 1448 bp *Xho*I to *Spe*I fragment of pNSE752.delta3'spl.sw/ha, which contains both the Swedish mutation and the Hardy mutation, to form PDAPP-Sw/Ha. A schematic of PDAPP-Sw/Ha and its construction is shown in Figure 13.

C. Construction of PDAPP695$_{V-F}$.

A construct encoding only APP695, but retaining the Hardy mutation, PDGF-B promoter, and vector sequences of PDAPP, can be made. This can be accomplished by ligating the 6.6 kb *Xho*I to *Nru*I fragment from PDAPP, which contains the C-terminal part of the APP sequences, and the polyadenylation, pUC, and PDGF-B promoter sequences, to the 1.2 kb *Xho*I to *Bcl*I fragment of pCK695, which contains a hybrid splice signal and the remaining N-terminal portion of the APP sequences (on a 911 bp *Xho*I to *Nru*I fragment of APP695 cDNA). The hybrid splice signal is the same as was described earlier and is also present in vector pohCK751, which is described by Dugan *et al.*, *J Biological Chem.* 270:10982-10989 (1995). pCK695 is identical to pohCK751 except that the herpes simples virus replication and packaging sequences of pohCK751 were removed, and the plasmid encodes APP695 instead of APP751.

In this vector the PDGF-B promoter drives the expression of APP695 containing the mutation of Val717 to Phe. The hybrid splice signal is included to potentially enhance expression. Additional vectors derived from this may be constructed which lack any splice signals, or into which other splice signals have been added to obtain this same function.

D. Construction of PDAPP751$_{V-F}$.

A construct encoding only APP751, but retaining the Hardy mutation, PDGF-B promoter, and vector sequences of PDAPP, can be made. This can be accomplished by ligating the 6.65 kb *Xho*I to *Kpn*I fragment of PDAPP, including part of the APP sequences, the polyadenylation signals, pUC and PDGF-B promoter sequences to the 1.0 kb *Kpn*I to *Xho*I fragment containing the remainder of the human APP751 cDNA sequences (nucleotides 57 to 1084 of SEQ ID NO:3) to make the intermediate plasmid PDAPPδsp751$_{V-F}$.

The 1.0 kb *Kpn*I to *Xho*I fragment encoding a portion of human APP751 can be obtained from the plasmid poCK751, which is identical to pohCK751 except that the herpes simplex viral sequences were removed.

To introduce splicing sequences, the first intron from PDAPP, which is intron Δ6, is then inserted into PDAPPδsp751$_{V-F}$ to make PDAPP751$_{V-F}$. To accomplish this, the 2,758 bp *Asp*718 to *Sca*I fragment of PDAPP containing exons 2 through 6, intron Δ6, and part of exon 7, is ligated to the 6,736 bp fragment obtained by complete digestion of PDAPPδsp751$_{V-F}$ with *Asp*718 and partial digestion with *Sca*I. This 6,736 bp fragment provides the remaining additional APP sequences (part of exon 1, the rest of exon 7, and exons 9 through 18), polyadenylation signals, pUC and PDGF-B promoter sequences. The resulting construct is referred to as PDAPP751$_{V-F}$.

In this vector the PDGF-B promoter drives the expression of APP751 containing the mutation of Val717 to Phe. One splice signal (derived from intron 6) is included to potentially enhance expression. Additional vectors derived from this may be constructed which lack any splice signals, or into which other splice signals have been added to obtain this same function.

E. Construction of PDAPP770$_{V-F}$.

A construct encoding only APP770, but retaining the Hardy mutation, PDGF-B promoter, and vector sequences of PDAPP, can be made. This can be accomplished by replacing the *Kpn*I to *Xho*I fragment of PDAPP751$_{V-F}$ containing APP exons 2-7 and a part of exon 9, with the *Kpn*I to *Xho*I fragment of APP770 cDNA, which contains exons 2-8 and a part of exon 9. This fragment corresponds to nucleotides 57 to 1140 of SEQ ID NO:5. The resulting construct is referred to as PDAPP770$_{V-F}$.

In this vector the PDGF-B promoter drives the expression of APP770 containing the mutation of Val717 to Phe. PDAPP770$_{V-F}$ contains the same intron sequences present in PDAPP751$_{V-F}$. Additional vectors derived from this may be constructed into which a splice signals have been added to obtain enhanced expression.

Example 8: Expression Levels of APP Expression Products in Brain Tissue of PDAPP Mice.

The PDAPP mouse line described in Example 6 was examined for the levels of several derivatives of the APP in hippocampal, cortical, and cerebellar brain regions of mice of various ages. Levels of APP cleaved at the beta-secretase site (APPβ) and APP containing at least 12 amino acids of Aβ (FLAPP+APPα; a mixture of APPα and full length APP (FLAPP)) were found to be nearly constant within a given brain region at all ages evaluated. The hippocampus expressed the highest level of all APP forms. In contrast, guanidine extractable levels of Aβ showed remarkable age-dependent increases in a manner that mirrored the amyloid plaque deposition observed immunohistochemically. Specifically, Aβ levels in hippocampus increased 17-fold by 8 months of age and 106-fold by 1 year of age, compared to that found in 4 month old animals. At 1 year of age Aβ constitutes approximately 1% of the total protein in hippocampus. The cerebral cortex also showed large increases in Aβ with age. In contrast, the mean level of Aβ in cerebellum across all age groups was comparatively low and unchanging.

Further analysis of the Aβ in these brains using an ELISA specific for $A\beta_{1-42}$ showed that this longer version made up 27% of the 19 pmoles/g of the Aβ present in the brains of young animals; this percentage increased to 97% of the 690 pmoles/g in 12 month old animals. The selective deposition of $A\beta_{1-42}$ and the spacial distribution of the Aβ deposits are further evidence that the pathological processes ongoing in the PDAPP transgenic mice parallel the human Alzheimer's diseased condition.

Levels of Aβ-containing proteins were measured through the use of ELISAs configured with antibodies specific to Aβ, $A\beta_{1-42}$, APP cleaved at the β-secretase site (Seubert *et al.* (1993)), and APP containing the first 12 amino acids of Aβ (FLAPP+APPα; a mixture of full length APP and α-secretase cleaved APP (Esch *et al.*)). Striking similarities in both the regional variation and depositing form of Aβ are noted between the mouse model and the human AD condition. The results also show that, because of

the magnitude and temporal predictability of Aβ deposition, the PDAPP mouse is a practical model in which to test agents that either inhibit the processing of APP to Aβ or retard Aβ amyloidosis.

A. Materials and Methods.

1. Brain Tissue Preparation.

The heterozygote transgenic (Line 109, Games *et al.*; Rockenstein *et al.*) and non-transgenic animals were anesthetized with Nembutol (1:5 solution in 0.9% saline) and perfused intracardially with ice cold 0.9% saline. The brain was removed and one hemisphere was prepared for immunohistochemical analysis, while four brain regions (cerebellum, hippocampus, thalamus, and cortex) were dissected from the other hemisphere and used for Aβ and APP measures.

To prepare tissue for ELISAs, each brain region was homogenized in 10 volumes of ice cold guanidine buffer (5.0 M guanidine-HCl, 50 mM Tris-Cl, pH 8.0) using a motorized pestle (Kontes). The homogenates were gently mixed on a Nutator for three to four hours at room temperature, then either assayed directly or stored at -20°C prior to quantitation of Aβ and APP. Preliminary experiments showed the analytes were stable to this storage condition.

2. Aβ Measurements.

The brain homogenates were further diluted 1:10 with ice-cold casein buffer (0.25% casein, phosphate buffered saline (PBS), 0.05% sodium azide, 20 μg/ml aprotinin, 5 mM EDTA pH 8.0, 10 μg/ml leupeptin), reducing the final concentration of guanidine to 0.5 M, before centrifugation (16,000 x g for 20 minutes at 4°C). The Aβ standards (1-40 or 1-42 amino acids) were prepared such that the final composition included 0.5 M guanidine in the presence of 0.1% bovine serum albumin (BSA).

The "total" Aβ sandwich ELISA consists of two monoclonal antibodies (mAb) to Aβ. The capture antibody, 266, is specific to amino acids 13-28 of Aβ (Seubert *et al.* (1992)); while the antibody 3D6, which is specific to amino acids 1-5 of Aβ, was biotinylated and served as the reporter antibody. The 3D6 biotinylation procedure employs the manufacturer's

(Pierce) protocol for NHS-biotin labeling of immunoglobulins except 100 mM sodium bicarbonate, pH 8.5 buffer was used. The 3D6 antibody does not recognize secreted APP or full-length APP but detects only Aβ species with amino terminal aspartic acid. The assay has a lower limit of sensitivity of approximately 50 pg/ml (11.4 pM) and showed no cross-reactivity to the endogenous murine Aβ peptide at concentrations up to 1 ng/ml.

The configuration of the $A\beta_{1-42}$-specific sandwich ELISA employs the mAb 21F12, which was generated against amino acids 33-42 of Aβ. The antibody shows less than 0.4% cross-reactivity with $A\beta_{1-40}$ in either ELISA or competitive radioimmunoassay (RIA). Biotinylated 3D6 is also the reporter antibody in this assay which has a lower limit of sensitivity of approximately 125 pg/ml (28.4 pM).

The 266 and 21F12 mAbs were coated at 10 μg/ml into 96-well immunoassay plates (Costar) overnight at room temperature. The plates were then aspirated and blocked with 0.25% human serum albumin in PBS buffer for at least 1 hour at room temperature, then stored desiccated at 4°C until use. The plates were rehydrated with wash buffer prior to use. The samples and standards were added to the plates and incubated at room temperature for 1 hour. The plates were washed at least 3 times with wash buffer (Tris buffered saline, 0.05% Tween 20) between each step of the assay.

The biotinylated 3D6, diluted to 0.5 μg/ml in casein assay buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was incubated in the well for 1 hour at room temperature. Avidin-HRP (Vector, Burlingame, CA), diluted 1:4000 in casein assay buffer, was added to the wells and incubated for 1 hour at room temperature. The colorimetric substrate (100 μl), Slow TMB-ELISA (Pierce), was added and allowed to react for 15 minutes, after which the enzymatic reaction is stopped with 25 μl of 2 N H_2SO_4. Reaction product was quantified using a Molecular Devices Vmax measuring the difference in absorbance at 450 nm and 650 nm.

3. APP ELISAs.

Two different APP assays were utilized. The first recognizes APPα and full length forms of APP (FLAPP+APPα), while the second recognizes

APPβ (APP ending at the methionine preceding the Aβ domain (Seubert *et al.* (1993)). The capture antibody for both the FLAPP+APPα and APPβ assays is 8E5, a monoclonal antibody raised to a bacterially expressed fusion protein corresponding to human APP amino acids 444-592 (Games *et al.*). The reporter mAb (2H3) for the FLAPP+APPα assay was generated against amino acids 1-12 of Aβ. The lower limit of sensitivity for the 8E5/2H3 assay is approximately 11 ng/ml (150 pM). For the APPβ assay, the polyclonal antibody 192 was used as the reporter. This antibody has the same specificity as antibody 92 (Seubert *et al.* (1993)), that is, it is specific to the carboxy-terminus of the β-secretase cleavage site of APP. The lower limit of sensitivity for the β-secretase 8E5/192 assay is approximately 43 ng/ml (600 pM).

For both APP assays, the 8E5 mAb was coated onto 96-well Costar plates as described above for 266. Purified recombinant secreted APPα (the APP751 form) and APP596 were the reference standards used for the FLAPP+APPα and APPβ assays, respectively. APP was purified as described previously (Esch *et al.*) and APP concentrations were determined by amino acid analysis. The 5 M guanidine brain homogenate samples were diluted 1:10 in specimen diluent for a final buffer composition of 0.5 M NaCl, 0.1% NP-40, 0.5 M guanidine. The APP standards for the respective assays were diluted into buffer of the same final composition as for the samples. The APP standards and samples were added to the plate and incubated for 1.5 hours at room temperature. The plates were thoroughly washed between each step of the assay with wash buffer. Reporter antibodies 2H3 and 192 were biotinylated following the same procedure as for 3D6 and were incubated with samples for 1 hour at room temperature. Streptavidin-alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hour at room temperature. The fluorescent substrate 4-methyl-umbellipheryl-phosphate, was added, and the plates read on a Cytofluor™ 2350 (Millipore) at 365 nm excitation and 450 nm emission.

4. Monoclonal Antibody Production.

The immunogens for 3D6, 21F12, and 2H3 were separately conjugated to sheep anti-mouse immunoglobulin (Jackson Immunoresearch Labs) using maleimidohexanoyl-N-hydroxysuccinimide (Pierce). A/J mice (Jackson Laboratories) were given intraperitoneal injections (IP) of 100 μg of the appropriate immunogen emulsified in Freund's complete adjuvant (Sigma) and two subsequent IP injections of 100 μg immunogen were given on a biweekly basis in Freund's incomplete adjuvant (Sigma). Two to three weeks after the third boost, the highest titer mouse of a given immunogen was injected intravenously and intraperitoneally with 50-100 μg of immunogen in PBS. Three days post injection, the spleen was removed, splenocytes were isolated and fused with SP2/0-Ag14 mouse myeloma cells. The hybridoma supernatants were screened for high affinity monoclonal antibodies by RIA as previously described (Seubert *et al.* (1992)). Purified monoclonal antibodies were prepared from ascites.

5. Immunohistochemistry.

The tissue from one brain hemisphere of each mouse was drop-fixed in 4% paraformaldehyde and post-fixed for three days. The tissue was mounted coronally and 40 μm sections were collected using a vibratome. The sections were stored in anti-freeze at -20°C prior to staining. Every sixth section, from the posterior part of the cortex through the hippocampus, was immunostained with biotinylated 3D6 at 4°C, overnight. The sections were then incubated with horseradish peroxidase avidin-biotin complex (Vector) and developed using 3,3'-diaminobenzidine (DAB) as the chromogen.

B. Results.

1. Aβ and APP Assays.

The FLAPP+APPα assay recognizes secreted APP including the first 12 amino acids of Aβ. Since the reporter antibody ($2H3_{1\text{-}12}$) is not specific to the alpha clip site occurring between Aβ amino acids 16 and 17 (Esch *et al.*), this assay also recognizes full length APP. Preliminary experiments using immobilized APP antibodies to the cytoplasmic tail of full length APP to

deplete the mixture suggest that approximately 30 to 40% of the FLAPP+APPα is full length. The APPβ assay recognizes only the APP clipped immediately amino-terminal to the Aβ region due to the specificity of the polyclonal reporter antibody 192 (Seubert *et al.* (1993)).

The specific nature of the Aβ immunoreactivity was further characterized as follows. Guanidine homogenates of brain (excluding cerebellum and brain stem) were subjected to size exclusion chromatography (Superose 12) and the resulting fractions analyzed using the total Aβ assay. Comparisons were made of 2, 4, and 12 month transgenic mouse brain homogenates and a non-transgenic mouse brain homogenate to which $A\beta_{1-40}$ had been spiked at a level roughly equal to that found in the 12 month old transgenic mice. The elution profiles of the transgenic brain homogenate were similar in that the peak fractions of Aβ immunoreactivity occurred in the same position, a single broad symmetric peak which was coincident with the immunoreactive peak of spiked $A\beta_{1-40}$. Attempts were then made to immunodeplete the Aβ immunoreactivity using resin bound antibodies against Aβ (mAb 266 against $A\beta_{13-28}$), the secreted forms of APP (mAb 8E5 against $APP_{444-592}$ of the APP695 form), the carboxy-terminus of APP (mAb 13G8 against $APP_{676-695}$ of the APP695 form), or heparin agarose. Only the 266 resin captured Aβ immunoreactivity, demonstrating that full length APP or carboxy-terminal fragments of APP are not contributing to the Aβ measurement. The $A\beta_{1-42}$ ELISA employs a capture antibody that recognizes $A\beta_{1-42}$ but not $A\beta_{1-40}$. The $A\beta_{1-42}$ assay, like the total Aβ assay, is not affected by the full length or carboxy-terminal forms of APP containing the Aβ region in the homogenates as shown by similar immunodepletion studies.

2. Total Aβ and APP Measures.

Table 5 shows the levels of total Aβ, FLAPP+APPα, and APPβ in the hippocampus, cortex, cerebellum, and thalamus of transgenic mice as a function of age. Each data point represents the mean value for each age group. The relative levels of FLAPP+APPα and APPβ in all four brain regions remain relatively constant over time. The hippocampus expresses the highest levels of FLAPP+APPα and APPβ followed by the thalamus, cortex,

and cerebellum, respectively. In the hippocampus, the levels of FLAPP+APPα are approximately 3.5 to 4.5-fold higher than APPβ at all ages. The mean value of all ages for FLAPP+APPα and APPβ assays in the hippocampus were 674 ($\pm$465) pmoles/gram and 175 ($\pm$11) pmoles/gram, respectively. From this it can be estimated that the pool of brain APP consists of approximately 50% APPα, 30% full length APP, and 20% APPβ.

TABLE 5. PDAPP Transgene Cohort Animal Data
Total Aβ & APP Measures in pmoles/gram of Brain Tissue.

AGE IN MONTHS	Aβ & APP FORM	CEREBELLUM	HIPPOCAMPUS	CORTEX	THALAMUS
2	Aβ	4.03 $\pm$1.08 (n=8)	35.41 $\pm$6.38 (n=8)	14.25 $\pm$2.27 (n=8)	6.41 $\pm$1.59 (n=8)
2	FLAPP+ APPα	ND	ND	ND	ND
2	APPβ	ND	ND	ND	ND
4	Aβ	4.10 $\pm$0.61 (n=14)	38.08 $\pm$6.51 (n=14)	15.95 $\pm$2.60 (n=14)	7.60 $\pm$1.52 (n=14)
4	FLAPP+ APPα	395 $\pm$120 (n=14)	703 $\pm$106 (n=14)	446 $\pm$70 (n=14)	6.37 $\pm$166 (n=14)
4	APPβ	78 $\pm$38 (n=14)	198 $\pm$30 (n-14)	126 $\pm$23 (n=14)	70 $\pm$17 (n=14)
6	Aβ	4.55 $\pm$1.38 (n=10)	87.48 $\pm$30.33 (n=10)	30.19 $\pm$8.33 (n=10)	8.34 $\pm$2.40 (n=10)
6	FLAPP+ APPα	403 $\pm$77 (n=10)	694 $\pm$107 (n=10)	506 $\pm$97 (n=10)	670 $\pm$156 (n=10)
6	APPβ	51 $\pm$87 (n=10)	194 $\pm$35 (n=10)	129 $\pm$25 (n=10)	56 $\pm$33 (n=10)
6.5	Aβ	5.42 $\pm$1.08 (n=10)	133.63 $\pm$57.10 (n=10)	33.27 $\pm$12.19 (n=10)	8.83 $\pm$1.19 (n=10)
6.5	FLAPP+ APPα	346 $\pm$74 (n=10)	580 $\pm$115 (n=10)	436 $\pm$63 (n=10)	553 $\pm$123 (n=10)
6.5	APPβ	27 $\pm$77 (n=10)	169 $\pm$41 (n=10)	108 $\pm$16 (n=10)	58 $\pm$22 (n=10)
7	Aβ	4.44 $\pm$0.56 (n=10)	200.77 $\pm$94.68 (n=10)	60.55 $\pm$27.13 (n=10)	8.94 $\pm$1.19 (n=10)

7	FLAPP+ APPα	378 ±70 (n=10)	656 ±73 (n=10)	469 ±62 (n=10)	604 ±107 (n=10)
7	APPβ	56 ±52 (n=10)	176 ±27 (n=10)	101 ±20 (n=10)	56 ±28 (n=10)
7.5	Aβ	5.14 ±1.39 (n=10)	461.35 ±345.95 (n=10)	81.839 ±53.00 (n=10)	10.84 ±5.22 (n=10)
7.5	FLAPP+ APPα	362 ±54 (n=10)	554 ±77 (n=10)	409 ±44 (n=10)	503 ±80 (n=10)
7.5	APPβ	20 ±58 (n=10)	168 ±27 (n=10)	118 ±21 (n=10)	57 ±22 (n=10)
8	Aβ	4.42 ±0.73 (n=13)	635.52 ±302.45 (n=13)	128.68 ±62.80 (n=13)	10.87 ±3.39 (n=13)
8	FLAPP+ APPα	386 ±52 (n=13)	660 ±102 (n=13)	494 ±87 (n=13)	672 ±150 (n=13)
8	APPβ	64 ±77 (n=13)	174 ±27 (n=13)	102 ±26 (n=13)	57 ±30 (n=13)
8.5	Aβ	5.54 ±1.11 (n=10)	633.11 ±363.14 (n=10)	118.39 ±59.91 (n=10)	13.96 ±7.34 (n=10)
8.5	FLAPP+ APPα	439 ±79 (n=10)	764 ±114 (n=10)	558 ±80 (n=10)	750 ±132 (n=10)
8.5	APPβ	28 ±59 (n=10)	185 ±34 (n=10)	108 ±42 (n=10)	47 ±28 (n=10)
9	Aβ	5.52 ±1.11 (n=10)	1512.39 ±624.286 (n=10)	254.83 ±105.927 (n=10)	19.46 ±8.99 (n=10)
9	FLAPP+ APPα	500 ±112 (n=10)	763 ±125 (n=10)	549 ±78 (n=10)	815 ±167 (n=10)
9	APPβ	4 ±83 (n=10)	169 ±25 (n=10)	121 ±32 (n=10)	49 ±26 (n=10)
10	Aβ	4.04 ±1.02 (n=11)	2182.21 ±1194.49 (n=11)	343.49 ±165.531 (n=11)	15.46 ±13.38 (n=11)
10	FLAPP+ APPα	452 ±130 (n=11)	678 ±93 (n=11)	491 ±102 (n=11)	693 ±166 (n=11)
10	APPβ	52 ±32 (n=11)	159 ±22 (n=11)	87 ±15 (n=11)	46 ±10 (n=11)
12	Aβ	3.26 ±0.35 (n=9)	4356.23 ±1666.44 (n=9)	691.17 ±360.93 (n=9)	18.08 ±13.50 (n=9)
12	FLAPP+ APPα	385 ±166 (n=10)	638 ±272 (n=10)	444 ±171 (n=10)	708 ±278 (n=10)

12	APPβ	41 ±29 (n=10)	134 ±47 (n=10)	76 ±31 (n=10)	35 ±19 (n=10)

ND = not determined

In contrast to APP levels, Aβ levels increased dramatically with age in the hippocampus and cortex. However, no such increase was noted in the cerebellum of the PDAPP transgenic mice, and only a moderate increase was seen in thalamus (Table 5). The increase of Aβ is greater in the hippocampus relative to the cortex, which also correlates with the 3D6 immunohistochemical results (see discussion below). Compared to the cortex levels of 4 month old mice, Aβ levels increase 10-fold by 8 months of age and 41-fold at 12 months old (660 ± 380 pmoles Aβ/gram tissue at age 12 months). The corresponding increases in Aβ observed in hippocampus are even more impressive, as the 8 month value is 15 times that at 4 months old and increases to 106-fold at 12 months old (4,040 ± 1750 pmoles Aβ/g tissue at 12 months). At 12 months of age, Aβ constitutes approximately 1% of protein in hippocampus of the PDAPP mice.

To see if the dramatic rise in brain Aβ concentration is due to amyloid deposition, we next visualized Aβ deposits immunohistochemically, using the opposite hemisphere of the same mice used for Aβ measurements. Notably, a parallel increase in Aβ plaque burden and Aβ level exists. These findings strongly argue that the rise in brain Aβ concentration determined by ELISA is due to the age-dependent amyloidosis.

3. $A\beta_{1-42}$ Measures in Transgenic Mouse Brain.

Concentrations of $A\beta_{1-42}$ in the cortex of transgenic mice were evaluated at different ages. As shown in Table 6, the percentage of Aβ which is $A\beta_{1-42}$ in the cortex of transgenic mice, also increases with age. The ELISA data suggest that $A\beta_{1-42}$ is preferentially depositing in the transgenic mice, and that the deposits detected by mAb 3D6 immunostaining are primarily $A\beta_{1-42}$.

Table 6. $A\beta_{1-42}$ Levels in the Cortex of Transgenic Brain.

Age (months)	$A\beta_{1-42}$ (pmoles/g)
4	4.71
8	75.65

10	247.43
12	614.53

4. Aβ Immunostaining in PDAPP Transgenic Brain.

Transgenic animals with Aβ values representing the mean Aβ value of the age group were used for 3D6 immunostaining. A progression of Aβ deposition is seen in the 4, 8, 10, and 12 months old animals. At four months of age, transgenic brains contained small, rare punctate deposits, 20 μm in diameter, that were only infrequently observed in the hippocampus and frontal and cingulate cortex. By eight months of age, these regions contained a number of thioflavin-positive Aβ aggregates that formed plaques as large as 150 μm in diameter. At ten months of age, many large Aβ deposits were found throughout the frontal and cingulate cortex, and the molecular layers of the hippocampus. The outer molecular layer of the dentate gyrus receiving perforant pathway afferents from the entorhinal cortex was clearly delineated by Aβ deposition. This general pattern was more pronounced by heavier Aβ deposition at one year of age. The anatomical localization of Aβ deposition is remarkably similar to that seen in Alzheimer's disease.

C. Discussion.

Aβ amyloidosis is an established diagnostic criteria of Alzheimer's disease (Mirra *et al.*, *Neurology* 41:479-486 (1991)) and is consistently seen in higher cortical areas as well as the hippocampal formation of the brain in affected subjects. It is believed that Aβ amyloidosis is a relatively early event in the pathogenesis of AD that subsequently leads to neuronal dysfunction and dementia through a complex cascade of events (Mann *et al.*, *Neurodegeneration* 1:201-215 (1992); Morris *et al.*, *Neurology* 46:707-719 (1996)). Various pathways of APP processing have been described (reviewed in Schenk *et al.*, *J. Med. Chem.* 38:4141-4154 (1995)) including the major α-secretase pathway where cleavage of APP occurs with Aβ (Esch *et al.*) and the amyloidogenic or β-secretase pathway where cleavage of APP occurs at the N-terminus of Aβ (Seubert *et al.* (1993)). Further cleavage of APP leads to the constitutive production of Aβ forms including those ending at position 40 ($A\beta_{1-40}$) or 42 ($A\beta_{1-42}$). ELISAs that detect specific APP products arising

from these individual pathways in the PDAPP mouse brain allow determination of whether differential processing of APP contributes to the regional or temporal specificity of amyloid formation and deposition.

Aβ amyloid deposition seen in the PDAPP mouse brain is highly age and region specific. Amyloid deposition begins at around 7 months of age, and by 12 months of age, amyloid deposition is very profound throughout the hippocampus and in the rostral region of the cortex. The age dependent increases in amyloid deposition correlate well with the dramatic rise in Aβ levels in these brain regions as measured by ELISA assay. An increase in Aβ is measurable by 7 months of age and by 10 months the hippocampus as 2180 pmoles/g of Aβ, a concentration equivalent to that of my cytoskeletal proteins and comparable to the levels found in the cortex of human AD brain (Gravina *et al.*, *J. Biol. Chem.* 270:7013-7016 (1995)). Aβ levels in the cerebellum, an unaffected brain region, still are at 4 pmoles/g -- essentially unchanged relative to the levels at 4 months of age -- again correlating with amyloid deposition measured by histological analyses. These results indicate that in aged PDAPP mice, brain Aβ levels reflects amyloid burden and therefore direct immunoassay measurement of brain Aβ levels can be used to test for compounds that reduce amyloid plaque burden.

In the PDAPP mouse, individuals suffering Down's Syndrome, and individuals with certain forms of FAD, overproduction of Aβ is almost certainly an accelerating factor not only in Aβ deposition but in subsequent neuropathology (Citron *et al.*, Mann *et al.*, Miller *et al.*, *Archives of Biochem. Biophys.* 301:41-52 (1993)). A comparison of the Aβ measurements seen in the PDAPP mouse with those reported for AD brain tissue reveals several striking similarities. For example, in the PDAPP mouse, the relative levels of Aβ peptide in hippocampus from young (2 months of age) versus old (10 months of age) mice is nearly a hundred fold. Similar findings were noted by Gravina *et al.* in comparing control brain tissue relative to that of AD. The rise in brain Aβ levels in the PDAPP mouse is rather pronounced between the ages of six to nine months of age. Again, this timecourse parallels, in an accelerated manner, that seen in

Down's Syndrome brain tissue, where amyloid deposition begins at approximately 30 years of age and increases substantially until approximately age 60 (Mann).

In summary, the above results show that a reproducible increase in measurable Aβ occurs in the brain tissue of the PDAPP mice and that this increase correlates with the severity of amyloid deposition. These findings indicate that these mice can be used to identify agents or compounds that pharmacologically reduce Aβ peptide production or affect its deposition.

Example 9: Behavioral Differences in PDAPP Transgenic Mice.

Alzheimer's disease is characterized by cognitive deficits including memory loss, and impairment of memory functions. To determine if the disclosed transgenic mice exhibit similar deficits, transgenic (TG) and non-transgenic (nTG) mice were evaluated for task performance in three types of maze apparatus used to test working and reference memory; the Y maze, the radial arm maze (RAM), and the water maze. The transgenic mice tested represent the fifth generation derived from the PDAPP mice described in Example 6. The Y maze and the radial arm maze are used to assess spontaneous alternation which is a function of working memory. For the Y maze task, the mouse is placed in the stem of a Y maze twice, each time allowing a choice entry into one of the arms. Entering both arms is a successful alternation, requiring memory of the previously entered arm, while entering the same arm on both trials is a failure. Chance performance is 50% alternation, that is, 50% of the mice alternate.

For the radial arm maze task, the mouse is placed at the center of a maze with multiple arms radiating from the center. In the testing described below, a radial eight-arm maze was used. Alternation performance is measured by allowing only eight entries, with the number of different arms entered being the measure of performance. The number of different-arm entries can be compared to the number of different-arm entries expected by chance, which is 5.25 (Spetch and Wilkie, "A program that stimulates random choices in radial arm mazes and similar choice situations" *Behavior Research Methods & Instrumentation* 12:377-378 (1980)). Performance

above chance, that is, above 5.25, requires memory of the previously entered arms.

The water maze used for the tests described below consists of a pool of water in which a submerged platform is placed. This hidden platform (HP) can be found by swimming mice either by chance (first trial) or through memory of positional clues visible from the tank (subsequent trials). Subject mice were trained in the hidden platform task according to standard procedures. Briefly, mice were first pretrained in a small pool (47 cm diameter, 20 cm platform), which teaches them how to navigate in water, that the platform is the goal, that there is no other escape, and that to find it they must resist their natural inclination to stay along the sides of the pool. They were then trained to find a single platform position in the hidden platform task using a larger pool and smaller platform (71 cm pool, 9 cm platform).

During the HP task, visual cues were located inside the pool (intramaze cues; black pieces of cardboard - circle, plus, or horizontal lines - located in three quadrants at the top of the wall, which was 38 cm high above water level), and various room cues were visible outside the pool (extramaze cues).

The mice assigned to the characterization cohort study were tested on the behavioral tasks described above over 3 days during the week or two before euthanasia. Their transgenic status was not known to the tester. Non-transgenic littermates were used for comparison. Each morning the subject mice were run in the Y maze and RAM as described above. They were then tested for general strength on the inclined plane (INP) test. For this, mice were placed in a 10-cm-wide runway lined with ridged plastic and elevated with the head up at 35°. The angle was then increased until the mouse slid off, and the angle was recorded. This was repeated three times each day. The average scores for the three days were calculated for each mouse for the Y maze (0=repeat, 1=alternate), RAM (number of different arms and time to finish, 10 minute limit), and INP (average of all nine trials). General activity was also rated on the first day of testing. Each mouse was observed

in the cage, and picked up and held. A mouse that remained calmly in the hand was scored 1, with progressively greater activity and reaction to handling scored up to 4.

Following the above tests each day, mice were tested in the water maze as described above. Briefly, mice were pretrained in a small pool to climb on a large submerged platform as their only means of escape from the water. They were then given six blocks of four trials each to learn the location of a small platform in a large pool. For analysis, all four trials within each block were averaged. The exception was the first hidden platform block, for which only the last three trials were averaged. The first trial was analyzed separately, because it is the only one for which platform location could not be known, and thus did not relate to spatial learning. It is thus used as a control for non-spatial factors, such as motivation and swimming speed. The performance effects between blocks were analyzed as a repeated measure for the hidden platform task. Standard analysis of variance (ANOVA) calculations were used to assess the significance of the results.

Results in the RAM show that TG performed significantly worse than nTG across all ages (Group effect: $p=0.00006$). The time to finish was also significantly different between TG and nTG mice (Group effect: $p=0.005$). The correlation between the time to finish and the number of arms chosen was small ($R= -0.15$, $p=0.245$ in each group). This suggests that the consistent impairment in the RAM is not accounted for by the increased time to complete the task taken by TG mice. Results in the Y maze were also significantly different for TG and nTG mice (Group effect: $p=0.011$). Validation studies performed on non-transgenic mice indicate that the Y maze is a less sensitive measure than the RAM.

Measures of strength (INP) and activity indicate no differences between TG and nTG mice. These are considered very rough measures, with only large differences being detectable. There was, however, a decrease in the activity score for all mice over time (Age effect: $p=0.070$). There was a difference in body weight, with TG weighing 8% less than controls (Group

effect: p=0.0003), primarily in female TG mice. However, this does not seem to have an effect on the results, as shown by the lack of any difference in strength (see above) or swimming speed (see below) between TG and nTG mice.

Results of the hidden platform task, considered here a test of reference memory, show a consistent difference between TG and nTG mice. ANOVA reveals that the effects of transgenic status (Group effect: p=0.00016) and trial blocks (Block effect: p<0.00001) are significant. The effect of transgenic status on performance is accounted for by slower performance by TG mice across all trial blocks and ages. Analysis of Trial 1 reveals an effect of transgenic status (Group effect: p=0.018), suggesting a difference in performance before learning has occurred. However, an analysis of covariance, with trial 1 as the covariate, still yields a significant deficit in TG mice (p=0.00051).

It was also possible that some physical differences between TG and nTG mice, rather than cognitive differences, could have been responsible for some of the performance differences seen in the water maze tasks. However, no significant difference in strength or activity was observed (see above). Another possibility considered was the effect of swimming speed on performance since a slower swimmer with equivalent cognitive ability would take longer to reach the platform. To test this, video tracking was used in the hidden platform task to measure the distance travelled to reach the platform (a measure of the amount of searching done by the mice which is related to cognitive ability), the swimming speed (a measure of physical ability unrelated to cognitive ability), and the amount of time need to find the platform (a measure of the combination of both the distance travelled and the swimming speed). This was done in older and younger mice than reported above, using three trials per block and no pretraining. The time needed to find the platform was significantly different in TG and nTG mice (Group effect: p<0.0005), with the TG mice taking longer. However, the swimming speed was not significantly different between TG and nTG mice (Group effect: p=0.879). Thus, the difference in time needed to find the

platform is likely to be due to a cognitive difference between TG and nTG mice. This is confirmed by measures of the distance travelled to find the platform. The TG mice travelled significantly further than the nTG mice before reaching the platform (Group effect: $p<0.0005$). These results indicate that the differences seen between TG and nTG mice in the time to reach the platform in the water maze tasks are due to differences in cognitive ability.

To test whether nTG mice retain a better memory of the platform location than TG mice, a probe trial was given immediately following hidden platform training in which the platform was removed. Video tracking was used to determine the number of crossings of the former platform location made by the mice relative to crossings of non-platform locations. There was a significant difference seen between the relative crossings of TG and nTG mice (Group effect: $p=0.006$). This is evidence that the nTG mice remember the former location of the platform better than TG mice.

It was also possible that the difference observed between TG and nTG mice in the time needed to reach the platform could have been influenced by differences in perception of the cues or motivational differences. To test this, TG and nTG mice were subjected to visible platform tasks in the water maze. For these tasks, a platform was placed in the pool so that it was visible above the water. Three different platforms were tested, a dark platform 25 mm above the surface (most visible), a gray platform 25 mm above the surface, and a dark platform 5 mm above the surface (both less visible). The results show no difference in the time to find the most visible platform between TG and nTG mice (Group effect: $p=0.403$). There was not any greater decrease in performance in TG mice when less visible platforms were used, suggesting that their vision was as good as nTG mice. These results indicate that perceptual and motivational differences do not influence the time to reach the platform in the water maze tasks described above.

Performance differences between TG and nTG mice were shown for RAM, Y maze, and water maze cognitive tasks in mice aged 4 to 8 months

(2 to 12 months for the water maze). All of these differences indicate, and are consistent with, cognitive deficits in the transgenic mice as a group. The various tasks combined to test working memory and reference memory, both of which are implicated in cognitive impairment observed in Alzheimer's victims.

Example 10: Detection and Measurement of Alzheimer's Disease Markers.

A. Detection and Measurement of GFAP.

Glial fibrillary acidic protein (GFAP), a marker which increases in AD brain tissue, was measured in the following manner. Tissue extracts were prepared from hippocampi of control and PDAPP transgenic mice, as described in Example 6, aged 14 months. Tissue was sonicated in 10 volumes (v/w) of 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml calpain inhibitor 1. Protein determinations were made on the extract and SDS-PAGE sample buffer added before boiling the samples for 5 minutes. SDS-PAGE was performed using 12.5 μg of protein of each sample loaded onto 10% Tris-glycine gels (Novex). The proteins were transferred to Pro-Blot PVDF membranes by standard methods. GFAP immunoreactive proteins were detected using an anti-GFAP antibody from Sigma (G9269) used at a dilution of 1:2,000. An increase in immunoreactivity in general was observed, and a smaller anti-GFAP reactive species was also found to increase substantially, in the transgenic animals. In non-transgenic animals, this approximately 40 kD fragment gave a mean densitometer signal of 142.47, while in the transgenic animals, it gave a mean densitometer signal of 591.51. This difference was significant, with a P value of 0.0286.

B. Detection of Gliosis.

Gliosis is one of the changes that is associated with the neuropathology of Alzheimer's disease. The isoquinoline carboxamide PK 11195 has been shown to be a preferential marker of the peripheral benzodiazepine sites associated with gliosis. These sites have been shown to be enhanced in several diseases and animal models associated with neuronal

damage and activated necroglia including stroke (Stephenson *et al.*, J. Neuroscience 15:5263-5274 (1991)) and Alzheimer's disease (Diorio *et al.*, Neurobiology of Aging 12:255-258 (1991)). In particular Diorio and colleagues have shown an approximate 200% increase in [^{3}H] PK 11195 binding in some brain regions of AD patients, such as the temporal cortex compared with age-matched controls. In this example, the brains from the PDAPP mouse, described in Example 6, were examined for qualitative and quantitative changes in the binding of [^{3}H] PK 11195 in order to correlate with the previously described AD disease pathology. Two different approaches were utilized; radioreceptor binding to homogenates of different brain regions and receptor autoradiography.

1. Methods.

For the homogenate binding studies, PDAPP mice were euthanised by cervical dislocation and the brains rapidly dissected on ice. Homogenates (10 mg/ml wet weight) of cerebral cortex, hippocampus and cerebellum were prepared in 50 mM Tris HCl, pH 7.4. 0.3, 1.0 and 3.0 nM [^{3}H] PK 11195 was incubated with these brain regions for 60 minutes at 23°C followed by rapid filtration over Whatman GF/B filters using a Brandell cell harvester. Non-specific binding was determined using 1 μM unlabelled PK 11195. Quantitation was performed by liquid scintillation spectrometry.

In the autoradiographic studies, PDAPP mice were euthanised using carbon dioxide, the brains removed and snap frozen in methyl butane/dry ice. The brains were sectioned in the coronal plane through the hippocampus. Twenty micron thick sections were mounted on glass slides and stored at -20°C. Sections were incubated at 1 hour at 23°C in 170 mM Tris-HCl, pH 7.4 containing 1 nM [^{3}H] PK 11195. Non-specific binding was determined using 1 μM unlabelled PK 11195. Incubations were terminated by rinsing sections twice for 5 minutes in ice-cold incubation buffer followed by a brief wash in ice-cold distilled water. Following rapid drying, sections were exposed to tritium Hyperfilm (Amersham International) for up to 5 weeks.

2. Results.

Four heterozygous transgenic mice 3-4 months of age were evaluated in the homogenate binding studies and compared with litter-mate controls. No significant differences were observed between any of the brain regions of the transgenic animals and their respective controls. [^{3}H] PK 1119 autoradiography was performed to compare binding in a 12-month old heterozygotic transgenic mouse with an aged-matched non-transgenic control. Preliminary results from an autoradiogram exposed for five weeks indicated that several plaque-like structures were labeled in the retrosplenial cortex of the transgenic mouse, a region that invariably contains Aβ deposits. The pattern of labeling corresponded to microglial cell or astrocytic clumps associated with plaques, rather than the more widespread pattern of astrocytosis or microgliosis in the hippocampal and cortical parenchyma. The non-transgenic mouse did not show this labeling pattern.

No changes were observed in the 3-4 month animals but some evidence for an increase in [^{3}H] PK 11195 binding was seen in the 12-month animal.

C. Detection and Measurement of Cholinergic Nerve Terminals.

A population of cholinergic neurones projecting to the forebrain have been shown to be selectively decreased in the postmortem brains of patients diagnosed with Alzheimer's disease. Hemicholinium-3 is a potent inhibitor of high affinity choline uptake and has been shown to be a good marker of cholinergic nerve terminals (Pascual *et al.*, *J Neurochem* 54:792-800 (1990)). The total number of high affinity choline uptake sites in PDAPP transgenic animals, which are described in Example 6, has been measured using both crude whole-brain preparations and homogenates from selective brain regions using the selective ligand [^{3}H]-Hemicholinium-3 ([^{3}H]HCh-3).

[^{3}H]-Hemicholinium-3 binding was determined using a modification of the methods described in Pascual *et al.* Mice were euthanised by asphyxiation with carbon dioxide and the brains rapidly removed and dissected on ice. The cortex, cerebellum, striatum and hippocampus were homogenized in 5 ml of 10 mM phosphate buffer without NaCl. Samples were spun at 17,000 x g for 10 minutes, and the pellets washed twice in 5 ml

10 mM PO_4 buffer. The final pellet was resuspended in 5 ml 1X phosphate buffered saline (PBS) to produce a protein concentration of 0.5 mg/ml. Brain regions were assayed in triplicate for high affinity choline uptake sites by the addition of [^{3}H]HCh-3 (3 nM final concentration). Following a 20 minute incubation, assays were terminated by rapid filtration through Whatman GF/B filters using a Brandell cell harvester and washing with PBS. Filters were transferred into scintillation vials, and specific binding estimated by liquid scintillation spectrometry.

D. Detection and Measurement of Sodium-Potassium ATPase.

Ouabain has been shown to bind specifically to high affinity sites in mammalian brain and that these sites correspond to a neuronal form of sodium-potassium ATPase (Na/K-ATPase; Hauger *et al.*, *J Neurochem* 44:1709-1715 (1985)). These sites have been shown to decrease in animal models of neurodegenerative diseases. Alzheimer's disease is characterized by massive neurodegeneration (DeLacoste and White, *Neurobiology of Aging* 4(1):1-16 (1993)).

In order to estimate the extent of neurodegeneration in the PDAPP mouse the binding of ouabain was determined in mouse brain homogenates. Methods were adapted from those described by Hauger *et al.* Brain tissue was homogenized in 100 mM Tris HCl, pH 7.4 containing 200 mM NaCl and 10 mM $MgCl_2$ and resuspended in assay buffer to produce a final concentration of 100μ g protein per assay. Specific binding was determined with 1 to 200 nM [^{3}H] ouabain in a solution of 5 mM ATP, 100 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, and 200 mM NaCl, incubated for 30 minutes at 37°C. Non specific binding was determined in the presence of 100 mM ouabain and the absence of ATP. Assays were terminated by rapid filtration over Whatman GF/B filters. Tubes were washed with ice cold 50 mM Tris HCl, pH 7.4, 15 mM KCl, 5 mM $MgCl_2$. Filters were transferred into scintillation vials, and specific binding estimated by liquid scintillation spectrometry.

Measurements of Na/K-ATPase and Mg-ATPase activity in brain tissue of PDAPP transgenic mice of various ages and in non-transgenic

control brain tissue. These results show some significant differences in activity between transgenic and non-transgenic samples in older mice. Mouse brain homogenates from 4, 8, and 12 month old PDAPP transgenic (TG) mice, and from non-transgenic (nTG) mice, were prepared and assayed generally as described above. The activity of Mg-ATPase was also determined. The results are shown in Tables 7 and 8.

Table 7. Na/K-ATPase Activity in PDAPP Transgenic Mouse Brain.

Age	Tissue	Na/K-ATPase rate (pmole Pi/mg protein/min)	% of nTG
4	TG hippocampus	2.57±0.62	88±14
4	nTG hippocampus	2.53±0.51	-
4	TG cortex	0.57±0.13	72±9
4	nTG cortex	0.77±0.17	-
4	TG cerebellum	1.39±0.17	85±14
4	nTG cerebellum	2.14±0.53	-
8	TG hippocampus	4.63±1.72	153±53
8	nTG hippocampus	2.87±0.41	-
8	TG cortex	1.16±0.08	121±23
8	nTG cortex	1.15±0.20	-
8	TG cerebellum	2.74±0.81	183±53
8	nTG cerebellum	1.47±0.13	-
12	TG hippocampus	1.66±0.36	58±9
12	nTG hippocampus	3.11±0.94	-
12	TG cortex	1.45±0.40	109±17
12	nTG cortex	1.60±0.46	-
12	TG cerebellum	1.43±0.32	74±7
12	nTG cerebellum	2.04±0.64	-

Table 8. Mg-ATPase Activity in PDAPP Transgenic Mouse Brain.

Age	Tissue	Mg-ATPase rate (pmole Pi/mg protein/min)	% of nTG
4	TG hippocampus	2.83±0.38	110±14
4	nTG hippocampus	2.44±0.54	-
4	TG cortex	1.74±0.14	92±5
4	nTG cortex	1.87±0.18	-
4	TG cerebellum	2.58±0.44	100±12
4	nTG cerebellum	2.59±0.35	-
8	TG hippocampus	3.28±0.69	99±22
8	nTG hippocampus	3.32±0.39	-
8	TG cortex	1.72±0.11	73±6
8	nTG cortex	2.40±0.31	-
8	TG cerebellum	3.19±0.49	113±15
8	nTG cerebellum	2.77±0.23	-
12	TG hippocampus	1.48±0.21	65±7
12	nTG hippocampus	2.33±0.46	-
12	TG cortex	1.60±0.30	76±7
12	nTG cortex	2.06±0.40	-
12	TG cerebellum	1.61±0.26	78±8
12	nTG cerebellum	1.93±0.99	-

The difference in Na/K-ATPase activity between transgenic and non-transgenic tissue is significant ($p < 0.05$) in the case of 12 month old cerebellum, and is highly significant ($p < 0.01$) in the case of 12 month old hippocampus. The difference in Mg-ATPase activity between transgenic and non-transgenic tissue is significant ($p < 0.05$) in the case of 8 and 12 month old cortex, and is highly significant ($p < 0.01$) in the case of 12 month old hippocampus.

E. *In situ* Hybridization with Probes to Neurotrophic Factors.

The use of *in situ* hybridization to detect and localize mRNAs for specific gene products is well documented in the literature (Lewis *et al.*, *Molecular Imaging in Neuroscience: A Practical Approach* (New York, Oxford University Press. 1st ed., 1-21, 1993), Lu and Gillett, *Cell Vision* 1(2):169-176 (1994), Sirinathsinghji and Dunnett, *Molecular Imaging in Neuroscience: A Practical Approach* (New York, Oxford University Press. 1st., ed. 43-67, 1993), Lawerence and Singer, *Nuc. Acids Res.* 13:1777-1799 (1985), Zeller and Rogers, *Current Protocols in Molecular Biology* (New York, John Wiley and Sons. 14.3.1-14.5.5, 1995)). For illustrative purposes, the specific example described below utilizes ^{35}S-radiolabeled oligodeoxyribonucleotide probes to detect BDNF mRNA in cryostat sectioned mouse brain from the PDAPP transgenic mouse described in Example 6 and non-transgenic control mice. However, ^{33}P-labeled radioactive DNA probes, as well as *in vitro* transcribed complementary RNA probes, could be used as well. Non radioactive probe labeling methods may also be used (Knoll, *Current Protocols in Molecular Biology* (New York, John Wiley and Sons. 14.7.1-14.7.14, 1995)). Additionally, the choice of tissue pre-treatment for hybridization with probe (for example, paraffin embedded sections) and post-hybridization washes depend on the method used, examples of which are described in the references cited above. Known and appropriate precautions against RNase contamination should be employed and are also discussed in the above references.

1. Tissue Preparation.

Freshly dissected whole brains, or sub-regions of interest, from transgenic or control mice at various developmental stages, or post-natal ages, are snap frozen in isopentane pre-equilibrated to -70°C. If desired, the animals may be perfused with PBS to eliminate circulating cells from brain prior to dissection. The brains are removed following 15 to 20 seconds immersion in isopentane, wrapped in aluminum foil, labeled appropriately, and stored at -80°C for sectioning. It should be noted that although the signal from *in situ* hybridization to cryostat sectioned tissues is more sensitive than to paraffin embedded sections, it is dependent upon the time from

dissection to hybridization. Frozen tissue is preferably analyzed by hybridization with probe within six weeks. RNA integrity in tissues declines beyond this time. Thus, if longer time periods between dissection and analysis are anticipated, the tissue should be fixed (see, for example, Lu and Gillett) before long term storage at -80°C.

Prior to sectioning, Probe-On-Plus glass slides (Fisher Scientific, Pittsburgh, PA) can be made RNAase free by overnight soaking in absolute ethanol, air dried briefly in a dust free environment, and baked at 180°C for a minimum of 4 hours. After cooling to room temperature, the slides are coated with 0.01% poly-lysine (prepared in DEPC treated H_2O) for approximately 5 seconds, and air dried in a dust free area. The coated slides can be stored for up to one month before use in a slide box with silica gel or drierite pellets.

For sectioning, the frozen brain stored at -80°C is transferred to a cryostat at -20°C, mounted onto a sectioning block, embedded in OCT.®, and allowed to equilibrate. The tissue is then cut into 7 to 14 μm thick sections using a sterilized microtome knife (treated with 70% EtOH in DEPC H_2O), and thaw mounted onto poly-lysine coated slides. The slides are kept at -20°C until the sectioning is complete. The sections are fixed and dehydrated by immersing the slides sequentially in the solutions noted below.

1. once in 4% paraformaldehyde, 1X PBS, pH 7.4, at 0°C for 5 minutes (this solution should be made fresh, and can be stored for up to 1 week at 4°C);
2. twice in 1X PBS, 2.5 minutes each time;
3. once in 50% EtOH in DEPC H_2O for 5 minutes;
4. once in 70% EtOH in DEPC H_2O for 5 minutes;
5. once in 95% EtOH in DEPC H_2O for 5 minutes;

The fixed sections are stored immersed in the 95% EtOH/DEPC H_2O solution at 4°C until use. If the sections are not fixed immediately, they may be stored at -80°C in the presence of drierite until use. In this case the sections are allowed to equilibrate to room temperature prior to the fixation/dehydration steps.

2. Probe Design and Preparation.

The sequence of the mouse BDNF mRNA/cDNA (accession #55573) is available from the Genbank database of Nucleic acid sequences (NCBI, Bethesda, MD). Anti-sense oligodeoxynucleotide probes against BDNF were designed using the primer select module of the DNAstar™ software package (Lasergene Inc., Madison, WI). Numerous other software packages, such Oligo® (NBI, Plymouth, MN), offer similar capabilities, and are also suitable. Candidate probes of 45 to 55 nucleotides length, approximately 50% G+C content, and hybridizing to the pre-cursor or mature peptide encoding regions of the BDNF mRNA were synthesized on an ABI 380B DNA synthesizer. As specificity controls, sense oligonucleotides corresponding to each probe were also synthesized. Using the convention that the first nucleotide in the BDNF coding region is position 1, the BDNF probes synthesized correspond to BDNF nucleotide positions 47 to 94 (probes 2710 & 2711), 158 to 203 (probes 2712 & 2713), 576 to 624 (probes 2714 & 2715), and 644 to 692 (probes 2716 & 2717). The even numbered oligonucleotides are probes for the sense strand, and the odd numbered oligonucleotides are probes for the anti-sense strand.

For radiolabeling, the probes are gel purified on denaturing acrylamide gels and reconstituted in H_2O using standard protocols (Sambrook *et al.*). The probes (30 to 35 ng, 2 pmoles) are labeled by 3' homopolymeric tailing using terminal deoxynucleotidyl transferase (Promega, Madison WI) and ^{35}S-dATP (1000 Ci/mmol, Amersham Inc.) according to the enzyme manufacturer's recommendation. The radiolabeled probes are purified by column chromatography on size exclusion mini-spin columns (Biospin-6, Biorad Inc., Hercules, CA). The specific activity of the probes is quantitated by scintillation counting. Typical specific activities of the probes ranged from 1 X 10^9 to 5 X 10^9 cpm/μg.

3. Tissue Hybridization and Post Hybridization Washes.

In preparation for hybridization, the desired number of slides are removed from storage under alcohol, and allowed to air dry thoroughly in the slide rack (approximately 1 hour). Meanwhile, the probe is heat denatured in

a boiling H_2O bath for 2 to 5 minutes, quick chilled in an ice/H_2O bath, and diluted in hybridization buffer (10% dextran sulfate, 50% deionized formamide, 4X SSC, 5X Dehardts, 100 μg/ml sheared salmon sperm DNA, 100 μg/ml polyadenylic acid) to a final concentration of 5 X 10^3 to 10 X 10^3 cpm/μl. DTT is added to a 10 mM final concentration.

For hybridization, 100 μl of diluted probe in hybridization buffer (corresponding to 0.5 X 10^6 to 1.0 X 10^6 cpm probe) is carefully applied to each section being hybridized with probe. The solution is gently spread over the section with a pipet tip to cover the entire section(s) on each slide. The slides containing probe are then placed in humidified hybridization chambers at 42°C for hybridization overnight with the probe. The hybridization chambers can be covered utility boxes, or acrylic boxes, with raised platforms to accommodate slides. The boxes are lined with filter paper (or paper towels), saturated in 4X SSC, 50% formamide, and humidified by pre-incubating them with closed lids in a 42°C incubator for 1 to 3 hours before the slides are placed inside them. Although cover slips can be placed on the sections after the hybridization buffer is applied, this is not necessary provided the hybridization chambers are adequately humidified during the procedure. If pre-hybridization is used to obtain a lower background, the sections may be incubated at 42°C under 50 μl hybridization buffer (minus probe) per section for 1 to 2 hours. After this time, an equal volume of hybridization buffer containing probe at twice the concentration described above is applied to each section, and hybridization is carried out as described above.

For washes, the slides are transferred from the hybridization chamber to a slide holder. The slides can be placed in the slide holder every 4th or 5th slot so as to allow adequate flow of wash solution over the surface of each section. This placement can significantly lowers background on the sections. A moderate flow rate of wash solution over the surface of the sections promotes removal of unhybridized probe, and consequently reduces background. This is best accomplished during the 55°C wash steps by suspending the slides in the slide holder, above a magnetic stir bar. The stir

bar is preferably placed approximately one inch under the slide holder. This can be done by hanging the slide holder(s) from pipets straddling the wash chamber. A large beaker, or a 4 to 6 inch deep Pyrex baking dish makes a suitable wash chamber. The wash chamber is placed on a hot-plate stirrer, the temperature setting of which is precalibrated to maintain the wash solution at 55°C during the procedure. The changes of wash solution are made using pre-equilibrated solution. Washes and post wash dehydration of the sections are carried out as follows:

1. twice in 1X SSC at room temperature for 5 minutes each time;
2. three times in 1X SSC at 55°C for 30 minutes each time;
3. once in 1X SSC at room temperature for 1 min.
4. once in 0.1X SSC at room temperature for 15 seconds;
5. once in ultra pure H_2O at room temperature for 15 seconds;
6. once in 50% EtOH for 15 seconds;
7. once in 70% EtOH for 15 seconds;
8. once in 95% EtOH for 15 seconds;

The sections are air dried thoroughly at room temperature for 2 hours, followed by 30 minutes at 55°C. The dried sections on the slides are placed in autoradiographic cassette and exposed to X-ray film (Hyperfilm, β-max, Amersham Inc., Arlington Heights, IL) at 4°C for 2 to 3 days to estimate the exposure time required under emulsion. The X-ray film is developed according to the manufacturers recommendation. The sections are coated with emulsion (Amersham LM-1, #RPN40) by dipping in emulsion at 42°C under appropriate safelight conditions. The emulsion coated slides are air dried on a cooled surface for approximately 30 minutes, and transferred to a plastic slide box containing a drying agent (drierite pellets). The seams of the box can be sealed with black tape, and the box wrapped in several layers of aluminum foil to ensure a light-tight enclosure. Following 2 to 4 hours at room temperature to finish the drying, the boxes are transferred to 4°C for autoradiographic exposure for 2 to 6 weeks. Prior to developing the emulsion coated slides, the box is removed from the refrigerator and allowed to equilibrate to room temperature for approximately 1 hour. The slides are

then developed according to the manufacturers instructions, air dried for 1 to 2 hours, and if desired, counterstained with the appropriate counterstain.

Modifications and variations of the making and testing of transgenic animal models for testing of Alzheimer's disease will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Athena Neurosciences, Inc.
(ii) TITLE OF INVENTION: Method For Identifying Alzheimer's Disease Therapeutics Using Transgenic Animal Models
(iii) NUMBER OF SEQUENCES: 10
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Patrea L. Pabst
(B) STREET: 2800 One Atlantic Center
1201 West Peachtree Street
(C) CITY: Atlanta
(D) STATE: GA
(E) COUNTRY: USA
(F) ZIP: 30309-3450
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/480,653
(B) FILING DATE: June 7, 1995
(C) CLASSIFICATION:
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: ANS101CIP
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (404)-873-8794
(B) TELEFAX: (404)-873-8795
(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2085 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1-2085
(D) OTHER INFORMATION: /function= "coding region for APP695."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1               5                  10                  15

GCG CTG GAG GTA CCC ACT GAT GGT AAT GCT GGC CTG CTG GCT GAA CCC       96
Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
             20                  25                  30

CAG ATT GCC ATG TTC TGT GGC AGA CTG AAC ATG CAC ATG AAT GTC CAG      144
Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
         35                  40                  45

AAT GGG AAG TGG GAT TCA GAT CCA TCA GGG ACC AAA ACC TGC ATT GAT      192
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
     50                  55                  60

ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA GTC TAC CCT GAA CTG      240
Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
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65                  70                  75                  80

CAG ATC ACC AAT GTG GTA GAA GCC AAC CAA CCA GTG ACC ATC CAG AAC      288
Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85                  90                  95

TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT GTG      336
Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT CTC      384
Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

GTT CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT TGC      432
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

GAA ACT CAT CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT GAG      480
Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

AAG AGT ACC AAC TTG CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA ATT      528
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

GAC AAG TTC CGA GGG GTA GAG TTT GTG TGT TGC CCA CTG GCT GAA GAA      576
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190

AGT GAC AAT GTG GAT TCT GCT GAT GCG GAG GAG GAT GAC TCG GAT GTC      624
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205

TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT GGG AGT GAA GAC AAA      672
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210                 215                 220

GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG GAA GAA GAA      720
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240

GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG GAA      768
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255

GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC ATT      816
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

GCC ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT CGA      864
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

GTT CCT ACA ACA GCA GCC AGT ACC CCT GAT GCC GTT GAC AAG TAT CTC      912
Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290                 295                 300

GAG ACA CCT GGG GAT GAG AAT GAA CAT GCC CAT TTC CAG AAA GCC AAA      960
Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305                 310                 315                 320

GAG AGG CTT GAG GCC AAG CAC CGA GAG AGA ATG TCC CAG GTC ATG AGA     1008
Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
                325                 330                 335

GAA TGG GAA GAG GCA GAA CGT CAA GCA AAG AAC TTG CCT AAA GCT GAT     1056
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AAG AAG GCA GTT ATC CAG CAT TTC CAG GAG AAA GTG GAA TCT TTG GAA      1104
Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
        355                 360                 365

CAG GAA GCA GCC AAC GAG AGA CAG CAG CTG GTG GAG ACA CAC ATG GCC      1152
Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
    370                 375                 380

AGA GTG GAA GCC ATG CTC AAT GAC CGC CGC CGC CTG GCC CTG GAG AAC      1200
Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385                 390                 395                 400

TAC ATC ACC GCT CTG CAG GCT GTT CCT CCT CGG CCT CGT CAC GTG TTC      1248
Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
                405                 410                 415

AAT ATG CTA AAG AAG TAT GTC CGC GCA GAA CAG AAG GAC AGA CAG CAC      1296
Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420                 425                 430

ACC CTA AAG CAT TTC GAG CAT GTG CGC ATG GTG GAT CCC AAG AAA GCC      1344
Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
        435                 440                 445

GCT CAG ATC CGG TCC CAG GTT ATG ACA CAC CTC CGT GTG ATT TAT GAG      1392
Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
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465                 470                 475                 480

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                485                 490                 495

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TAC GGA AAC GAT GCT CTC ATG CCA TCT TTG ACC GAA ACG AAA ACC ACC      1584
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        595                 600                 605
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 695 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
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Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
65                  70                  75                  80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
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Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
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Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
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Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
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Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
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                245                 250                 255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290                 295                 300

Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
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Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
                325                 330                 335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
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Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385                 390                 395                 400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
                405                 410                 415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420                 425                 430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
        435                 440                 445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
    450                 455                 460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465                 470                 475                 480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
                485                 490                 495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
            500                 505                 510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
        515                 520                 525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
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Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
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Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
        595                 600                 605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    610                 615                 620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625                 630                 635                 640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
                645                 650                 655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
            660                 665                 670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
        675                 680                 685

Phe Phe Glu Gln Met Gln Asn
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2253 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1-2253
(D) OTHER INFORMATION: /function= "coding region for APP751."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
            20                  25                  30

CAG ATT GCC ATG TTC TGT GGC AGA CTG AAC ATG CAC ATG AAT GTC CAG      144
Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
        35                  40                  45

AAT GGG AAG TGG GAT TCA GAT CCA TCA GGG ACC AAA ACC TGC ATT GAT      192
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
    50                  55                  60

ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA GTC TAC CCT GAA CTG      240
Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
65                  70                  75                  80

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                85                  90                  95
```

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TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT GTG      336
Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT CTC      384
Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

GTT CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT TGC      432
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

GAA ACT CAT CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT GAG      480
Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

AAG AGT ACC AAC TTG CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA ATT      528
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

GAC AAG TTC CGA GGG GTA GAG TTT GTG TGT TGC CCA CTG GCT GAA GAA      576
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190

AGT GAC AAT GTG GAT TCT GCT GAT GCG GAG GAG GAT GAC TCG GAT GTC      624
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205

TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT GGG AGT GAA GAC AAA      672
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210                 215                 220

GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG GAA GAA GAA      720
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240

GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG GAA      768
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255

GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC ATT      816
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

GCC ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT CGA      864
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

GAG GTG TGC TCT GAA CAA GCC GAG ACG GGG CCG TGC CGA GCA ATG ATC      912
Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
    290                 295                 300

TCC CGC TGG TAC TTT GAT GTG ACT GAA GGG AAG TGT GCC CCA TTC TTT      960
Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
305                 310                 315                 320

TAC GGC GGA TGT GGC GGC AAC CGG AAC AAC TTT GAC ACA GAA GAG TAC     1008
Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
                325                 330                 335

TGC ATG GCC GTG TGT GGC AGC GCC ATT CCT ACA ACA GCA GCC AGT ACC     1056
Cys Met Ala Val Cys Gly Ser Ala Ile Pro Thr Thr Ala Ala Ser Thr
            340                 345                 350

CCT GAT GCC GTT GAC AAG TAT CTC GAG ACA CCT GGG GAT GAG AAT GAA     1104
Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp Glu Asn Glu
        355                 360                 365
```

```
CAT GCC CAT TTC CAG AAA GCC AAA GAG AGG CTT GAG GCC AAG CAC CGA      1152
His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala Lys His Arg
    370                 375                 380

GAG AGA ATG TCC CAG GTC ATG AGA GAA TGG GAA GAG GCA GAA CGT CAA      1200
Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala Glu Arg Gln
385                 390                 395                 400

GCA AAG AAC TTG CCT AAA GCT GAT AAG AAG GCA GTT ATC CAG CAT TTC      1248
Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile Gln His Phe
                405                 410                 415

CAG GAG AAA GTG GAA TCT TTG GAA CAG GAA GCA GCC AAC GAG AGA CAG      1296
Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn Glu Arg Gln
            420                 425                 430

CAG CTG GTG GAG ACA CAC ATG GCC AGA GTG GAA GCC ATG CTC AAT GAC      1344
Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met Leu Asn Asp
        435                 440                 445

CGC CGC CGC CTG GCC CTG GAG AAC TAC ATC ACC GCT CTG CAG GCT GTT      1392
Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu Gln Ala Val
    450                 455                 460

CCT CCT CGG CCT CGT CAC GTG TTC AAT ATG CTA AAG AAG TAT GTC CGC      1440
Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys Tyr Val Arg
465                 470                 475                 480

GCA GAA CAG AAG GAC AGA CAG CAC ACC CTA AAG CAT TTC GAG CAT GTG      1488
Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe Glu His Val
                485                 490                 495

CGC ATG GTG GAT CCC AAG AAA GCC GCT CAG ATC CGG TCC CAG GTT ATG      1536
Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser Gln Val Met
            500                 505                 510

ACA CAC CTC CGT GTG ATT TAT GAG CGC ATG AAT CAG TCT CTC TCC CTG      1584
Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser Leu Ser Leu
        515                 520                 525

CTC TAC AAC GTG CCT GCA GTG GCC GAG GAG ATT CAG GAT GAA GTT GAT      1632
Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp Glu Val Asp
    530                 535                 540

GAG CTG CTT CAG AAA GAG CAA AAC TAT TCA GAT GAC GTC TTG GCC AAC      1680
Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val Leu Ala Asn
545                 550                 555                 560

ATG ATT AGT GAA CCA AGG ATC AGT TAC GGA AAC GAT GCT CTC ATG CCA      1728
Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala Leu Met Pro
                565                 570                 575

TCT TTG ACC GAA ACG AAA ACC ACC GTG GAG CTC CTT CCC GTG AAT GGA      1776
Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro Val Asn Gly
            580                 585                 590

GAG TTC AGC CTG GAC GAT CTC CAG CCG TGG CAT TCT TTT GGG GCT GAC      1824
Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe Gly Ala Asp
        595                 600                 605

TCT GTG CCA GCC AAC ACA GAA AAC GAA GTT GAG CCT GTT GAT GCC CGC      1872
Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val Asp Ala Arg
    610                 615                 620

CCT GCT GCC GAC CGA GGA CTG ACC ACT CGA CCA GGT TCT GGG TTG ACA      1920
Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr
625                 630                 635                 640
```

```
AAT ATC AAG ACG GAG GAG ATC TCT GAA GTG AAG ATG GAT GCA GAA TTC      1968
Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp Ala Glu Phe
                645                 650                 655

CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG GTG TTC TTT      2016
Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe
            660                 665                 670

GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT GGA CTC ATG GTG      2064
Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val
        675                 680                 685

GGC GGT GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC TTG GTG ATG CTG      2112
Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
    690                 695                 700

AAG AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT GTG GTG GAG GTT GAC      2160
Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp
705                 710                 715                 720

GCC GCT GTC ACC CCA GAG GAG CGC CAC CTG TCC AAG ATG CAG CAG AAC      2208
Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn
                725                 730                 735

GGC TAC GAA AAT CCA ACC TAC AAG TTC TTT GAG CAG ATG CAG AAC          2253
Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
            740                 745                 750
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 751 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1               5                  10                  15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
             20                  25                  30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
         35                  40                  45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
     50                  55                  60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65                  70                  75                  80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                 85                  90                  95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
```

```
                 165                 170                 175
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210                 215                 220
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285
Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
    290                 295                 300
Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
305                 310                 315                 320
Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
                325                 330                 335
Cys Met Ala Val Cys Gly Ser Ala Ile Pro Thr Thr Ala Ala Ser Thr
            340                 345                 350
Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp Glu Asn Glu
        355                 360                 365
His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala Lys His Arg
    370                 375                 380
Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala Glu Arg Gln
385                 390                 395                 400
Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile Gln His Phe
                405                 410                 415
Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn Glu Arg Gln
            420                 425                 430
Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met Leu Asn Asp
        435                 440                 445
Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu Gln Ala Val
    450                 455                 460
Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys Tyr Val Arg
465                 470                 475                 480
Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe Glu His Val
                485                 490                 495
Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser Gln Val Met
            500                 505                 510
Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser Leu Ser Leu
        515                 520                 525
```

```
Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp Glu Val Asp
    530                 535                 540

Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val Leu Ala Asn
545                 550                 555                 560

Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala Leu Met Pro
                565                 570                 575

Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro Val Asn Gly
            580                 585                 590

Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe Gly Ala Asp
        595                 600                 605

Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val Asp Ala Arg
    610                 615                 620

Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr
625                 630                 635                 640

Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp Ala Glu Phe
                645                 650                 655

Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe
            660                 665                 670

Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val
        675                 680                 685

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
    690                 695                 700

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp
705                 710                 715                 720

Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn
                725                 730                 735

Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
            740                 745                 750
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2310 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
- (A) NAME/KEY: CDS
- (B) LOCATION: 1-2310
- (D) OTHER INFORMATION: /function= "coding region for APP770."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATG CTG CCC GGT TTG GCA CTG CTC CTG CTG GCC GCC TGG ACG GCT CGG       48
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1               5                  10                  15

GCG CTG GAG GTA CCC ACT GAT GGT AAT GCT GGC CTG CTG GCT GAA CCC       96
Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
             20                  25                  30

CAG ATT GCC ATG TTC TGT GGC AGA CTG AAC ATG CAC ATG AAT GTC CAG      144
Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
```

```
        35                  40                  45

AAT GGG AAG TGG GAT TCA GAT CCA TCA GGG ACC AAA ACC TGC ATT GAT      192
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
     50                  55                  60

ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA GTC TAC CCT GAA CTG      240
Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65                  70                  75                  80

CAG ATC ACC AAT GTG GTA GAA GCC AAC CAA CCA GTG ACC ATC CAG AAC      288
Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                 85                  90                  95

TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT GTG      336
Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT CTC      384
Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

GTT CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT TGC      432
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

GAA ACT CAT CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT GAG      480
Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

AAG AGT ACC AAC TTG CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA ATT      528
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

GAC AAG TTC CGA GGG GTA GAG TTT GTG TGT TGC CCA CTG GCT GAA GAA      576
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190

AGT GAC AAT GTG GAT TCT GCT GAT GCG GAG GAG GAT GAC TCG GAT GTC      624
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205

TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT GGG AGT GAA GAC AAA      672
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210                 215                 220

GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG GAA GAA GAA      720
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240

GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG GAA      768
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255

GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC ATT      816
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

GCC ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT CGA      864
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

GAG GTG TGC TCT GAA CAA GCC GAG ACG GGG CCG TGC CGA GCA ATG ATC      912
Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
    290                 295                 300

TCC CGC TGG TAC TTT GAT GTG ACT GAA GGG AAG TGT GCC CCA TTC TTT      960
```

```
Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
305                 310                 315                 320

TAC GGC GGA TGT GGC GGC AAC CGG AAC AAC TTT GAC ACA GAA GAG TAC     1008
Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
                325                 330                 335

TGC ATG GCC GTG TGT GGC AGC GCC ATG TCC CAA AGT TTA CTC AAG ACT     1056
Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr
            340                 345                 350

ACC CAG GAA CCT CTT GCC CGA GAT CCT GTT AAA CTT CCT ACA ACA GCA     1104
Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala
        355                 360                 365

GCC AGT ACC CCT GAT GCC GTT GAC AAG TAT CTC GAG ACA CCT GGG GAT     1152
Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp
    370                 375                 380

GAG AAT GAA CAT GCC CAT TTC CAG AAA GCC AAA GAG AGG CTT GAG GCC     1200
Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala
385                 390                 395                 400

AAG CAC CGA GAG AGA ATG TCC CAG GTC ATG AGA GAA TGG GAA GAG GCA     1248
Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala
                405                 410                 415

GAA CGT CAA GCA AAG AAC TTG CCT AAA GCT GAT AAG AAG GCA GTT ATC     1296
Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile
            420                 425                 430

CAG CAT TTC CAG GAG AAA GTG GAA TCT TTG GAA CAG GAA GCA GCC AAC     1344
Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn
        435                 440                 445

GAG AGA CAG CAG CTG GTG GAG ACA CAC ATG GCC AGA GTG GAA GCC ATG     1392
Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met
    450                 455                 460

CTC AAT GAC CGC CGC CGC CTG GCC CTG GAG AAC TAC ATC ACC GCT CTG     1440
Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu
465                 470                 475                 480

CAG GCT GTT CCT CCT CGG CCT CGT CAC GTG TTC AAT ATG CTA AAG AAG     1488
Gln Ala Val Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys
                485                 490                 495

TAT GTC CGC GCA GAA CAG AAG GAC AGA CAG CAC ACC CTA AAG CAT TTC     1536
Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe
            500                 505                 510

GAG CAT GTG CGC ATG GTG GAT CCC AAG AAA GCC GCT CAG ATC CGG TCC     1584
Glu His Val Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser
        515                 520                 525

CAG GTT ATG ACA CAC CTC CGT GTG ATT TAT GAG CGC ATG AAT CAG TCT     1632
Gln Val Met Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser
    530                 535                 540

CTC TCC CTG CTC TAC AAC GTG CCT GCA GTG GCC GAG GAG ATT CAG GAT     1680
Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp
545                 550                 555                 560

GAA GTT GAT GAG CTG CTT CAG AAA GAG CAA AAC TAT TCA GAT GAC GTC     1728
Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val
                565                 570                 575
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```
TTG GCC AAC ATG ATT AGT GAA CCA AGG ATC AGT TAC GGA AAC GAT GCT      1776
Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala
            580                 585                 590

CTC ATG CCA TCT TTG ACC GAA ACG AAA ACC ACC GTG GAG CTC CTT CCC      1824
Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro
        595                 600                 605

GTG AAT GGA GAG TTC AGC CTG GAC GAT CTC CAG CCG TGG CAT TCT TTT      1872
Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe
    610                 615                 620

GGG GCT GAC TCT GTG CCA GCC AAC ACA GAA AAC GAA GTT GAG CCT GTT      1920
Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val
625                 630                 635                 640

GAT GCC CGC CCT GCT GCC GAC CGA GGA CTG ACC ACT CGA CCA GGT TCT      1968
Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser
                645                 650                 655

GGG TTG ACA AAT ATC AAG ACG GAG GAG ATC TCT GAA GTG AAG ATG GAT      2016
Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp
            660                 665                 670

GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG      2064
Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
        675                 680                 685

GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT GGA      2112
Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly
    690                 695                 700

CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC TTG      2160
Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu
705                 710                 715                 720

GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT GTG GTG      2208
Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val
                725                 730                 735

GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC CAC CTG TCC AAG ATG      2256
Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met
            740                 745                 750

CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG TTC TTT GAG CAG ATG      2304
Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met
        755                 760                 765

CAG AAC                                                             2310
Gln Asn
    770
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 770 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1               5                  10                  15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
            20                  25                  30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
        35                  40                  45
```

```
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
     50                  55                  60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65                  70                  75                  80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85                  90                  95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210                 215                 220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
    290                 295                 300

Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
305                 310                 315                 320

Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
                325                 330                 335

Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr
            340                 345                 350

Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala
        355                 360                 365

Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp
    370                 375                 380

Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala
385                 390                 395                 400

Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala
```

405 410 415

Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile
420 425 430

Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn
435 440 445

Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met
450 455 460

Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu
465 470 475 480

Gln Ala Val Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys
485 490 495

Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe
500 505 510

Glu His Val Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser
515 520 525

Gln Val Met Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser
530 535 540

Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp
545 550 555 560

Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val
565 570 575

Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala
580 585 590

Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro
595 600 605

Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe
610 615 620

Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val
625 630 635 640

Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser
645 650 655

Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp
660 665 670

Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
675 680 685

Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly
690 695 700

Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu
705 710 715 720

Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val
725 730 735

Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met
740 745 750

Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met
755 760 765

Gln Asn
770

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCGATGATGA CGAGGACGAT 20

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGAACACGTG ACGAGGCCGA 20

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Phe Arg Val Gly Ser
5

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Ala Glu Phe Arg Gly Gly Cys
5

We claim:

1. A method for testing compounds for an effect on an Alzheimer's disease marker comprising

a) administering the compound to be tested to a non-human transgenic mammal, or mammalian cells derived from the transgenic mammal, wherein the transgenic mammal has a nucleic acid construct stably incorporated into the genome, wherein the construct comprises a promoter for expression of the construct in a mammalian cell and a region encoding an Aβ-containing protein, wherein the promoter is operatively linked to the region,

wherein the region comprises DNA encoding the Aβ-containing protein, wherein the Aβ-containing protein consists of all or a contiguous portion of a protein selected from the group consisting of

APP770, APP770 bearing a mutation in one or more of the amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, and 717, APP751, APP751 bearing a mutation in one or more of the amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, and 717, APP695, and APP695 bearing a mutation in one or more of the amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, and 717,

wherein the Aβ-containing protein includes amino acids 672 to 714 of human APP,

wherein the promoter mediates expression of the construct such that $A\beta_{tot}$ is expressed at a level of at least 30 nanograms per gram of brain tissue of the mammal when it is two to four months old, $A\beta_{1-42}$ is expressed at a level of at least 8.5 nanograms per gram of brain tissue of the mammal when it is two to four months old, APP and APPα combined are expressed at a level of at least 150 picomoles per gram of brain tissue of the mammal when it is two to four months old, APPβ is expressed at a level of at least 40 picomoles per gram of brain tissue of the mammal when it is two to four months old, and/or mRNA encoding the Aβ-containing protein is expressed to a level at least twice that of mRNA encoding the endogenous APP of the transgenic mammal in brain tissue of the mammal when it is two to four months old; and

detecting or measuring the Alzheimer's disease marker such that any difference between the marker in the transgenic mammal, or by mammalian cells derived from the transgenic mammal, and the marker in a transgenic mammal, or by mammalian cells derived therefrom, to which the compound has not been administered, is observed,

wherein an observed difference in the marker indicates that the compound has an effect on the marker.

2. The method of claim 1 wherein the Aβ-containing protein is selected from the group consisting of APP770; APP770 bearing a mutation in the codon encoding one or more amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, 717; APP751; APP751 bearing a mutation in the codon encoding one or more amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, 717; APP695; APP695 bearing a mutation in the codon encoding one or more amino acids selected from the group consisting of amino acid 669, 670, 671, 690, 692, 717; a protein consisting of amino acids 646 to 770 of APP; a protein consisting of amino acids 670 to 770 of APP; a protein consisting of amino acids 672 to 770 of APP; and a protein consisting of amino acids 672 to 714 of APP.

3. The method of claim 2 wherein the DNA encoding the Aβ-containing protein is cDNA or a cDNA/genomic DNA hybrid, wherein the cDNA/genomic DNA hybrid includes at least one APP intron sequence wherein the intron sequence is sufficient for splicing.

4. The method of claim 1 wherein the promoter is the human platelet derived growth factor β chain gene promoter.

5. The method of claim 1 wherein the region further comprises DNA encoding a second protein, wherein the DNA encoding the Aβ-containing protein and the DNA encoding the second protein are operative linked such that the region encodes an Aβ-containing fusion protein comprising a fusion of the Aβ-containing protein and the second protein.

6. The method of claim 5 wherein the second protein is a signal peptide.

7. The method of claim 1 wherein the Alzheimer's disease marker is a protein and the observed difference is an increase or decrease in the amount of

the protein present in the transgenic mammal, or in mammalian cells derived therefrom, to which the compound has been administered.

8. The method of claim 7 wherein the protein is selected from the group consisting of Cat D,B, Neuronal Thread Protein, nicotine receptors, 5-HT_2 receptor, NMDA receptor, α2-adrenergic receptor, synaptophysin, p65, glutamine synthetase, glucose transporter, PPI kinase, GAP43, cytochrome oxidase, heme oxygenase, calbindin, adenosine A1 receptors, choline acetyltransferase, acetylcholinesterase, glial fibrillary acidic protein (GFAP), α1-antitrypsin, C-reactive protein, α2-macroglobulin, IL-1α, IL-1β, TNFα, IL-6, HLA-DR, HLA-A, D,C, CR3 receptor, MHC I, MHC II, CD 31, CR4, CD45, CD64, CD4, spectrin, tau, ubiquitin, MAP-2, apolipoprotein E, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), advanced glycosylation end products, receptor for advanced glycosylation end products, COX-2, CD18, C3, fibroblast growth factor, CD44, ICAM-1, lactotransferrin, C1q, C3d, C4d, C5b-9, gamma RI, Fc gamma RII, CD8, CD59, vitronectin, vitronectin receptor, beta-3 integrin, Apo J, clusterin, type 2 plasminogen activator inhibitor, midkine, macrophage colony stimulating factor receptor, MRP14, 27E10, interferon-alpha, S100β, $cPLA_2$, c-jun, c-fos, HSP27, HSP70, MAP5, membrane lipid peroxidase, protein carbonyl formation, junB, junD, fosB, fra1, cyclin D1, p53, NGFI-A, NGFI-B, IκB, NFκB, IL-8, MCP-1, MIP-1α, matrix metaloproteinases, 4-hydroxynonenal-protein conjugates, amyloid P component, laminin, and collagen type IV.

9. The method of claim 1 wherein the Alzheimer's disease marker is a protein and the observed difference is a reduction or absence of the protein in plaques or neuritic tissue present in the transgenic mammal to which the compound has been administered.

10. The method of claim 9 wherein the protein is selected from the group consisting of Cat D,B, protein kinase C, NADPH, C3d, C1q, C5, C4bp, C5a-C9, tau, ubiquitin, MAP-2, neurofilaments, heparin sulfate, chrondroitin sulphate, apolipoprotein E, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glycosylation end products, amyloid P component, laminin, and collagen type IV.

11. The method of claim 1 wherein the Alzheimer's disease marker is a protein and the observed difference is an increase or decrease in the enzymatic or biochemical activity of the protein in the transgenic mammal, or in mammalian cells derived therefrom, to which the compound has been administered.

12. The method of claim 11 wherein the protein is selected from the group consisting of nicotine receptors, 5-HT_2 receptor, NMDA receptor, α2-adrenergic receptor, glutamine synthetase, glucose transporter, PPI kinase, cytochrome oxidase, heme oxygenase, calbindin, adenosine A1 receptors, choline acetyltransferase, acetylcholinesterase, glial fibrillary acidic protein (GFAP), α1-antitrypsin, C-reactive protein, α2-macroglobulin, IL-1, TNFα, IL-6, HLA-DR, HLA-A, D,C, CR3 receptor, MHC I, MHC II, CD 31, CR4, CD45, CD64, CD4, spectrin, ubiquitin, and apolipoprotein E.

13. The method of claim 1 wherein the Alzheimer's disease marker is a nucleic acid encoding a protein and the observed difference is an increase or decrease in the amount of the nucleic acid present in the transgenic mammal, or in mammalian cells derived therefrom, to which the compound has been administered.

14. The method of claim 13 wherein the encoded protein is selected from the group consisting of growth inhibitory factor, Cat D,B, Neuronal Thread Protein, nicotine receptors, 5-HT_2 receptor, NMDA receptor, α2-adrenergic receptor, synaptophysin, p65, glutamine synthetase, glucose transporter, PPI kinase, GAP43, cytochrome oxidase, heme oxygenase, calbindin, adenosine A1 receptors, choline acetyltransferase, acetylcholinesterase, glial fibrillary acidic protein (GFAP), α1-antitrypsin, C-reactive protein, α2-macroglobulin, IL-1, TNFα, IL-6, HLA-DR, HLA-A, D,C, CR3 receptor, MHC I, MHC II, CD 31, CR4, CD45, CD64, CD4, spectrin, tau, ubiquitin, MAP-2, apolipoprotein E, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), advanced glycosylation end products, receptor for advanced glycosylation end products, COX-2, CD18, C3, fibroblast growth factor, CD44, ICAM-1, lactotransferrin, C1q, C3d, C4d, C5b-9, gamma RI, Fc gamma RII, CD8, CD59, vitronectin, vitronectin receptor, beta-3 integrin, Apo J, clusterin, type 2 plasminogen activator inhibitor, midkine, macrophage colony stimulating factor receptor,

MRP14, 27E10, interferon-alpha, S100β, cPLA$_2$, c-jun, c-fos, HSP27, HSP70, MAP5, membrane lipid peroxidase, protein carbonyl formation, junB, junD, fosB, fra1, cyclin D1, p53, NGFI-A, NGFI-B, IκB, NFκB, IL-8, MCP-1, MIP-1α, matrix metaloproteinases, 4-hydroxynonenal-protein conjugates, amyloid P component, laminin, and collagen type IV.

15. The method of claim 1 wherein the Alzheimer's disease marker is a behavior and the observed difference is a change in the behavior observed in the transgenic mammal to which the compound has been administered.

16. The method of claim 15 wherein the behavior is selected from the group consisting of behavior using working memory, behavior using reference memory, locomotor activity, emotional reactivity to a novel environment or to novel objects, and object recognition.

17. The method of claim 1 wherein the Alzheimer's disease marker is a histopathology and the observed difference is a decrease in the extent or severity of the histopathology present in the transgenic mammal to which the compound has been administered.

18. The method of claim 17 wherein the histopathology marker is selected from the group consisting of compacted plaques, neuritic dystrophy, gliosis, Aβ deposits, decreased synaptic density, and neuropil abnormalities.

19. The method of claim 1 wherein the Alzheimer's disease marker is cognition and the observed difference is a change in the cognition of the transgenic mammal to which the compound has been administered.

20. The method of claim 1 wherein the marker is detected or measured using RT-PCR, RNase protection, Northern analysis, R-dot analysis, ELISA, antibody staining, laser scanning confocal imaging, and immunoelectron micrography.

21. The method of claim 1 wherein the mammals are rodents.

22. The method of claim 1 wherein the codon encoding amino acid 717 is mutated to encode an amino acid selected from the group consisting of Ile, Phe, Gly, Tyr, Leu, Ala, Pro, Trp, Met, Ser, Thr, Asn, and Gln.

23. The method of claim 22 wherein the codon encoding amino acid 717 is mutated to encode Phe.

24. The method of claim 1 wherein the codon encoding amino acid 670 is mutated to encode an amino acid selected from the group consisting of Asn and Glu, or the codon encoding amino acid 670 is deleted, and/or

wherein the codon encoding amino acid 671 is mutated to encode an amino acid selected from the group consisting of Ile, Leu, Tyr, Lys, Glu, Val, and Ala, or the codon encoding amino acid 671 is deleted.

25. The method of claim 24 wherein the codon encoding amino acid 670 is mutated to encode Asn, and/or the codon encoding amino acid 671 is mutated to encode Leu or Tyr.

26. The method of claim 1 wherein the promoter mediates expression of the construct such that $A\beta_{tot}$ is expressed at a level of at least 30 nanograms per gram of hippocampal or cortical brain tissue of the mammal when it is two to four months old, $A\beta_{1-42}$ is expressed at a level of at least 8.5 nanograms per gram of hippocampal or cortical brain tissue of the mammal when it is two to four months old, APP and APPα combined are expressed at a level of at least 150 picomoles per gram of hippocampal or cortical brain tissue of the mammal when it is two to four months old, APPβ is expressed at a level of at least 40 picomoles per gram of hippocampal or cortical brain tissue of the mammal when it is two to four months old, and/or mRNA encoding the Aβ-containing protein is expressed to a level at least twice that of mRNA encoding the endogenous APP of the transgenic mammal in hippocampal or cortical brain tissue of the mammal when it is two to four months old.

27. The method of claim 1 wherein amyloid plaques that can be stained with Congo Red are present in brain tissue of the mammal.

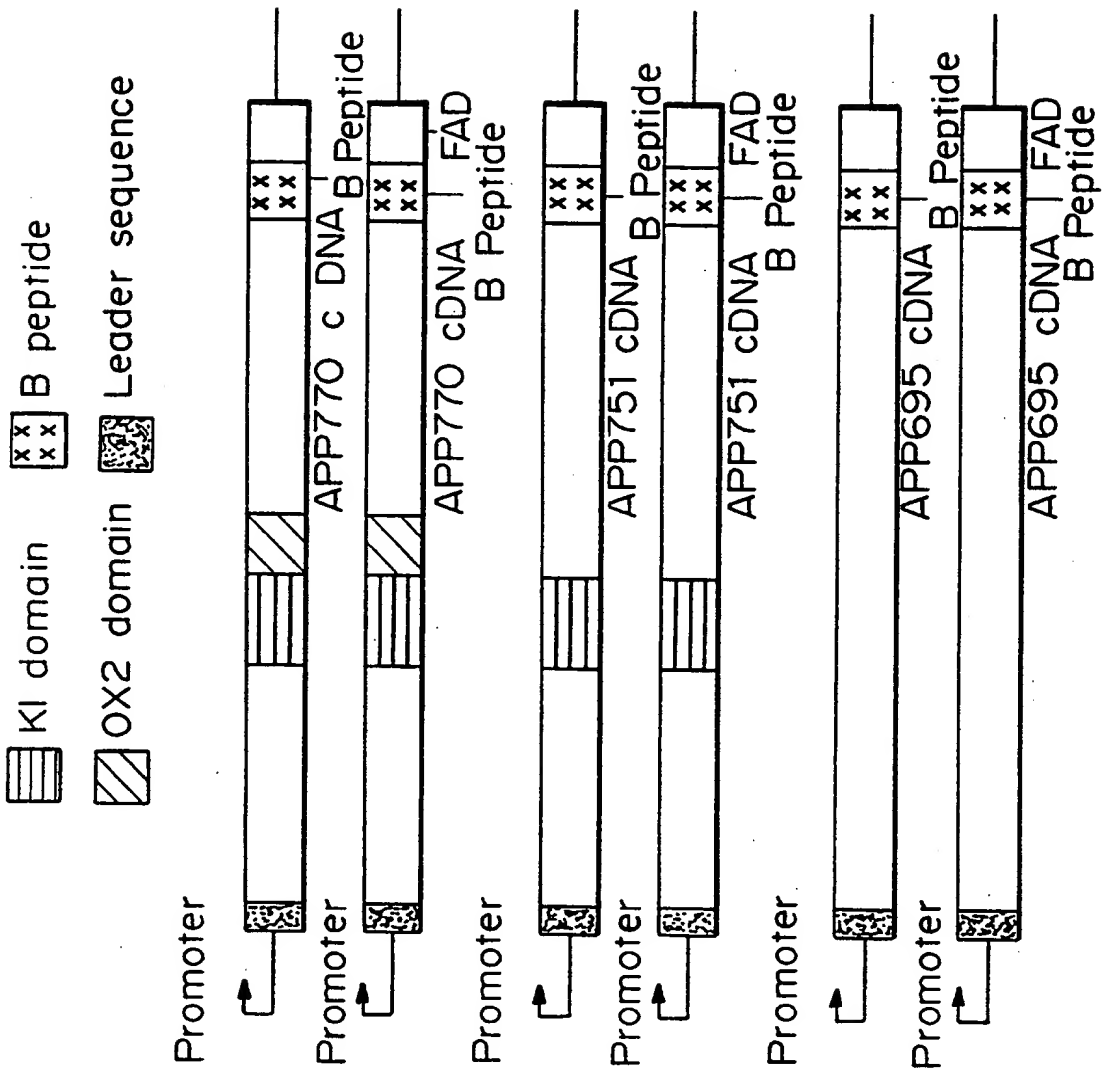


FIG. 1a

FIG. 1b

FIG. 2a

FIG. 2b

FIG. 3a

FIG. 3b

FIG. 4a

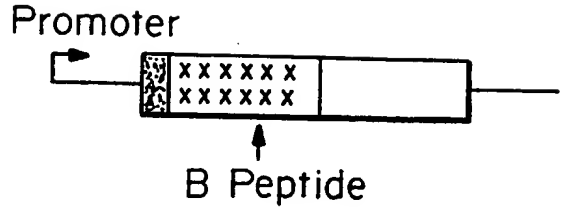


FIG. 4b

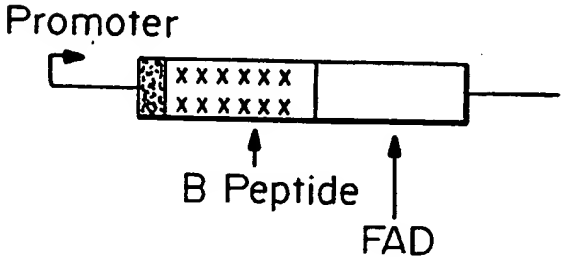
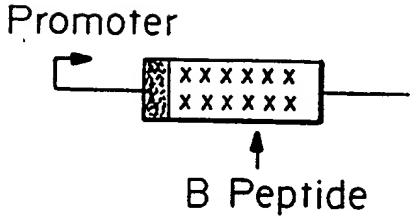


FIG. 5



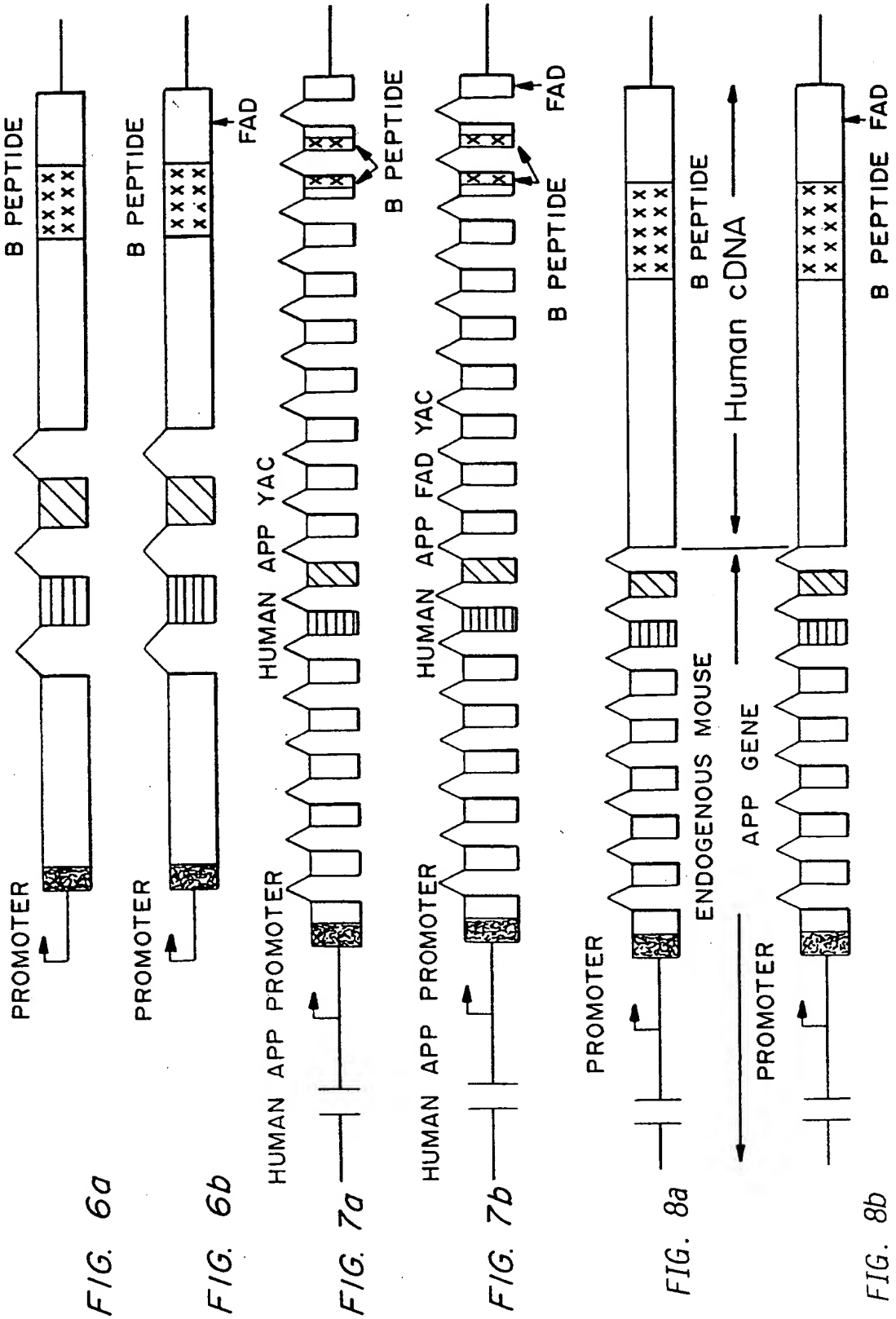


FIG. 6a FIG. 6b FIG. 7a FIG. 7b FIG. 8a FIG. 8b

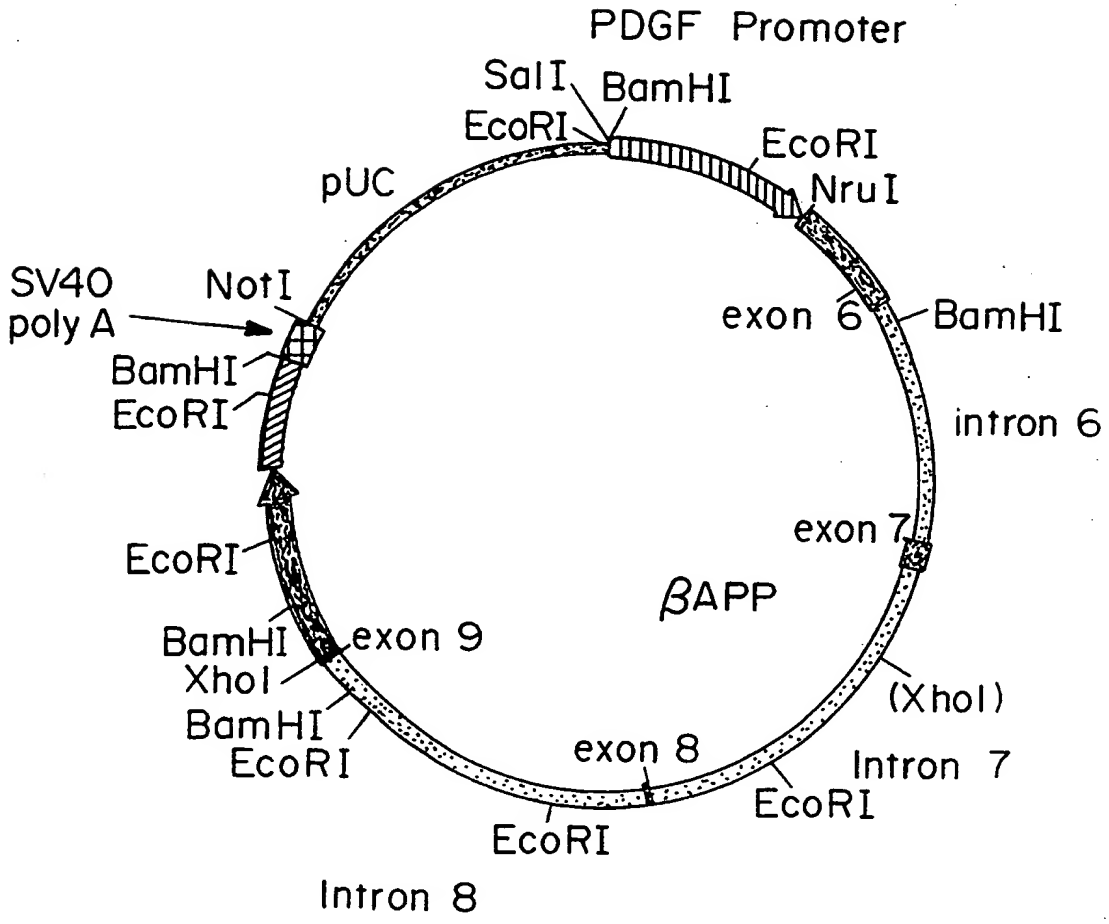
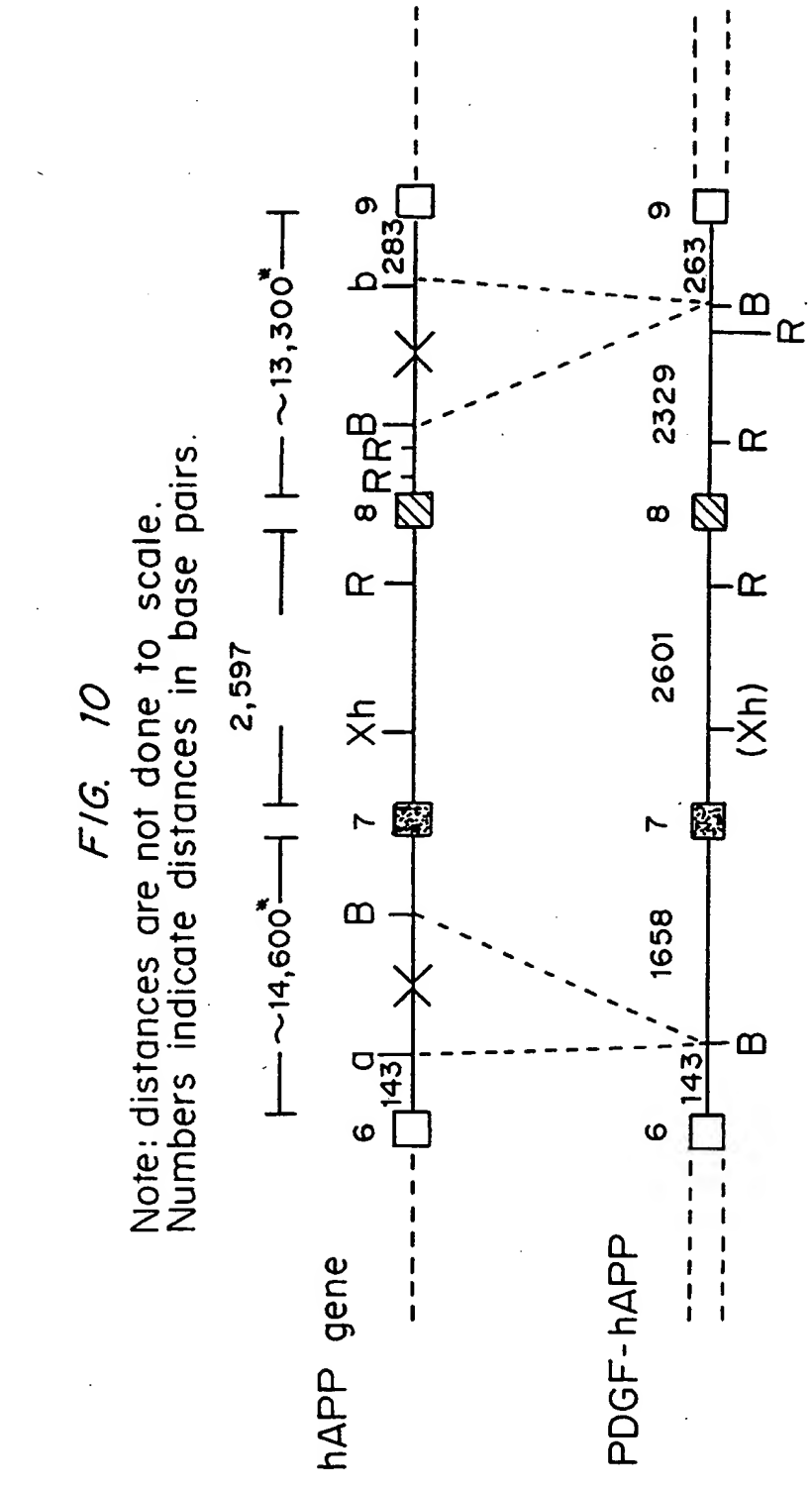

PDGF Promoter
SalI
BamHI
EcoRI
EcoRI
NruI
pUC
SV40
poly A
NotI
exon 6
BamHI
BamHI
EcoRI
intron 6
exon 7
EcoRI
βAPP
BamHI
exon 9
XhoI
(XhoI)
BamHI
EcoRI
exon 8
Intron 7
EcoRI
EcoRI
Intron 8

FIG. 9

FIG. 10

Note: distances are not done to scale.
Numbers indicate distances in base pairs.



a, b - sites of mutagenesis where BamHI sites were engineered for deletion
B = location of BamHI sites
X = XhoI, (x) XhoI site destroyed
R = EcoRI
Intron 7 was expanded by 4 b.p. when the XhoI site was destroyed.

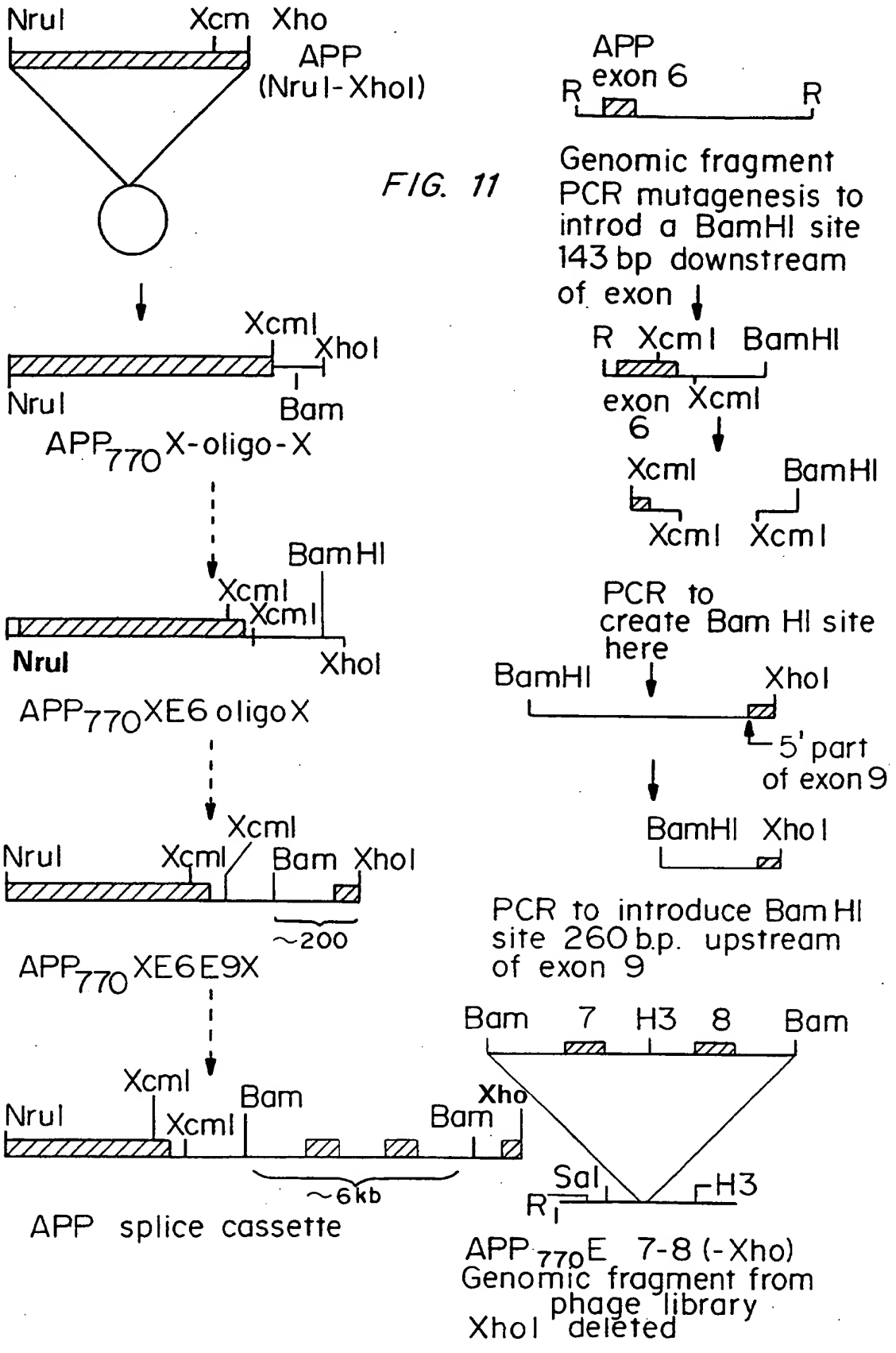
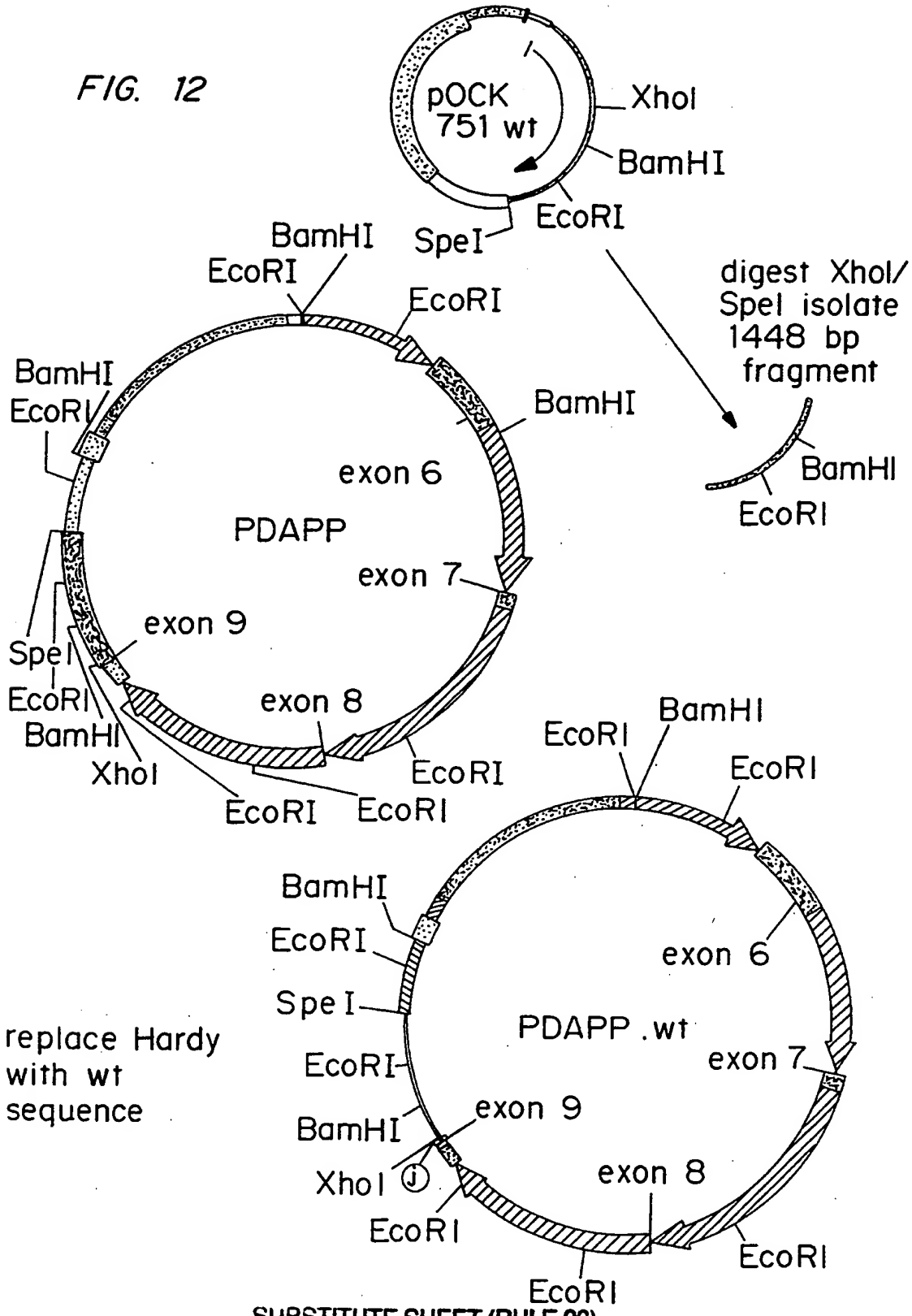
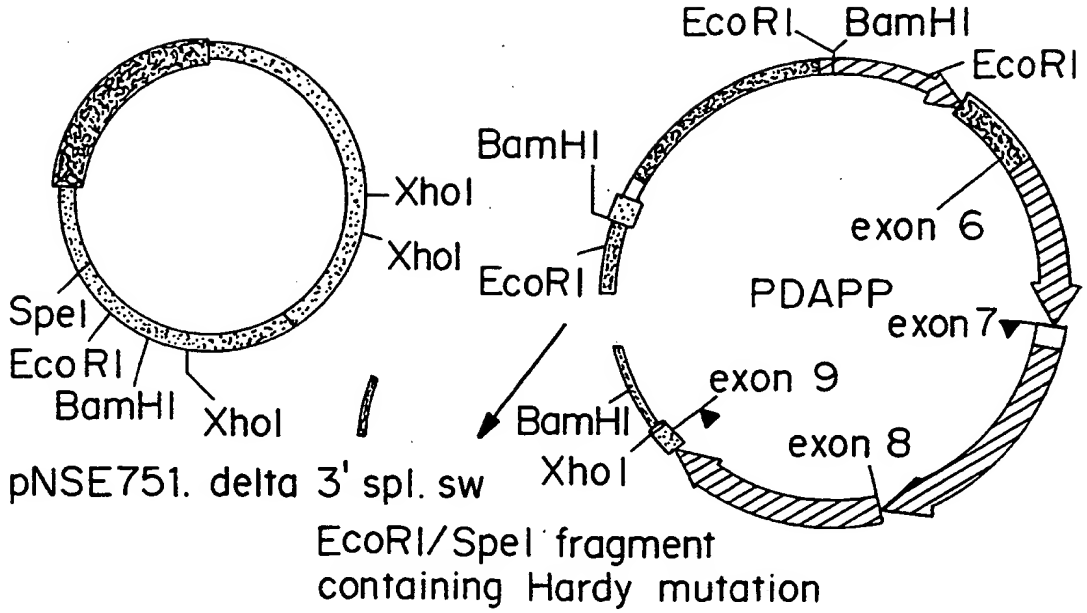
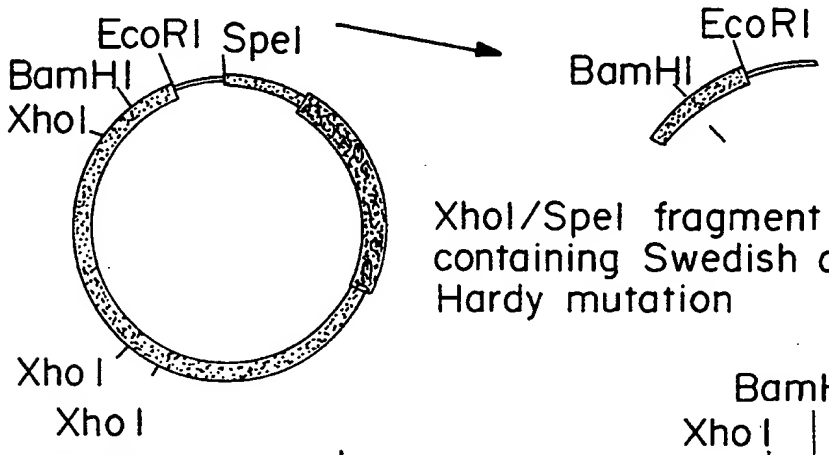

NruI
Xcm
Xho
APP
(NruI-XhoI)
XcmI
XhoI
NruI
Bam
APP770X-oligo-X
BamHI
XcmI
XcmI
NruI
XhoI
APP770XE6 oligoX
XcmI
NruI
XcmI
Bam
XhoI
~200
APP770XE6E9X
XcmI
NruI
XcmI
Bam
Bam
Xho
~6kb
APP splice cassette
APP
exon 6
R
R
Genomic fragment
PCR mutagenesis to
introd a BamHI site
143 bp downstream
of exon
R
XcmI
BamHI
exon
6
XcmI
XcmI
BamHI
XcmI
XcmI
PCR to
create Bam HI site
here
BamHI
XhoI
5' part
of exon 9
BamHI
XhoI
PCR to introduce Bam HI
site 260 b.p. upstream
of exon 9
Bam
7
H3
8
Bam
Xho
Sal
H3
R
APP770E 7-8 (-Xho)
Genomic fragment from
phage library
XhoI deleted

FIG. 11

FIG. 12


pOCK
751 wt
XhoI
BamHI
EcoRI
SpeI
digest XhoI/
SpeI isolate
1448 bp
fragment
BamHI
EcoRI
BamHI
EcoRI
EcoRI
BamHI
EcoRI
BamHI
exon 6
PDAPP
exon 7
exon 9
SpeI
EcoRI
BamHI
XhoI
exon 8
EcoRI
EcoRI
EcoRI
BamHI
EcoRI
EcoRI
BamHI
EcoRI
SpeI
EcoRI
BamHI
XhoI
exon 6
PDAPP .wt
exon 7
exon 9
exon 8
EcoRI
EcoRI
EcoRI
replace Hardy
with wt
sequence


EcoRI
BamHI
EcoRI
BamHI
XhoI
XhoI
exon 6
EcoRI
PDAPP
exon 7
SpeI
EcoRI
BamHI
XhoI
exon 9
BamHI
XhoI
exon 8
pNSE751. delta 3' spl. sw
EcoRI/SpeI fragment
containing Hardy mutation


EcoRI
SpeI
BamHI
XhoI
EcoRI
BamHI
XhoI/SpeI fragment
containing Swedish and
Hardy mutation
XhoI
XhoI
pNSE751. delta 3' spl. sw/ha
(9823 bp)

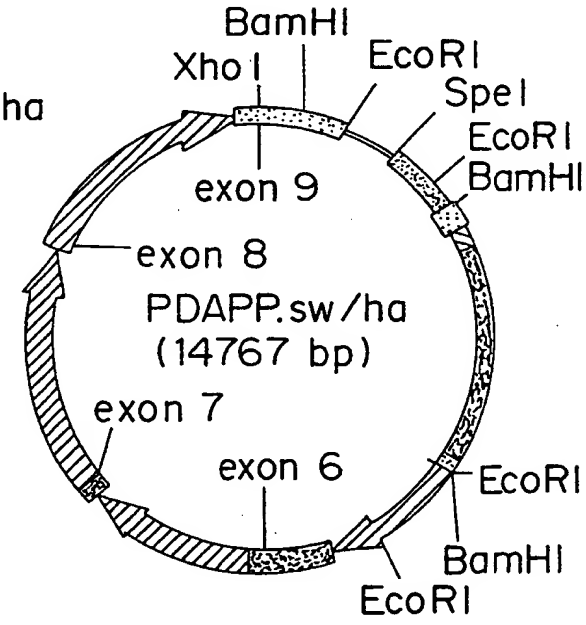

BamHI
XhoI
EcoRI
SpeI
EcoRI
BamHI
exon 9
exon 8
PDAPP.sw/ha
(14767 bp)
exon 7
exon 6
EcoRI
BamHI
EcoRI

FIG. 13

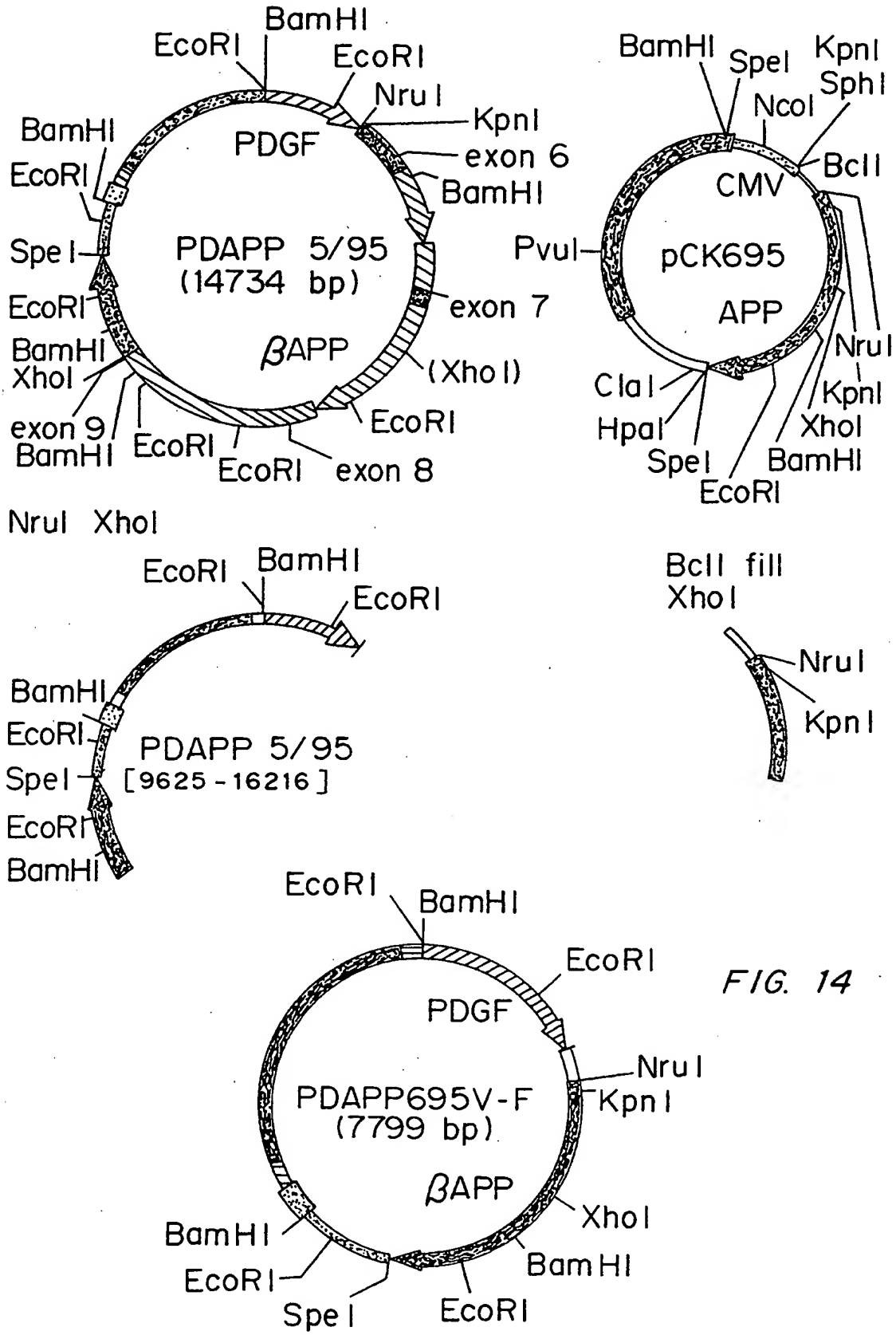

BamHI
EcoRI
EcoRI
Nru I
Kpn I
exon 6
BamHI
PDGF
PDAPP 5/95
(14734 bp)
βAPP
exon 7
(Xho I)
EcoRI
EcoRI
EcoRI
exon 8
BamHI
EcoRI
Spe I
EcoRI
BamHI
Xho I
exon 9
BamHI
Nru I Xho I
BamHI Spe I Kpn I
Sph I
Nco I
Bcl I
CMV
Pvu I
pCK695
APP
Nru I
Kpn I
Xho I
BamHI
EcoRI
Spe I
Cla I
Hpa I
Bcl I fill
Xho I
Nru I
Kpn I
EcoRI BamHI
EcoRI
BamHI
EcoRI
PDAPP 5/95
[9625 - 16216]
Spe I
EcoRI
BamHI
EcoRI BamHI
EcoRI
PDGF
Nru I
Kpn I
PDAPP695V-F
(7799 bp)
βAPP
Xho I
BamHI
BamHI
EcoRI
Spe I
EcoRI

FIG. 14

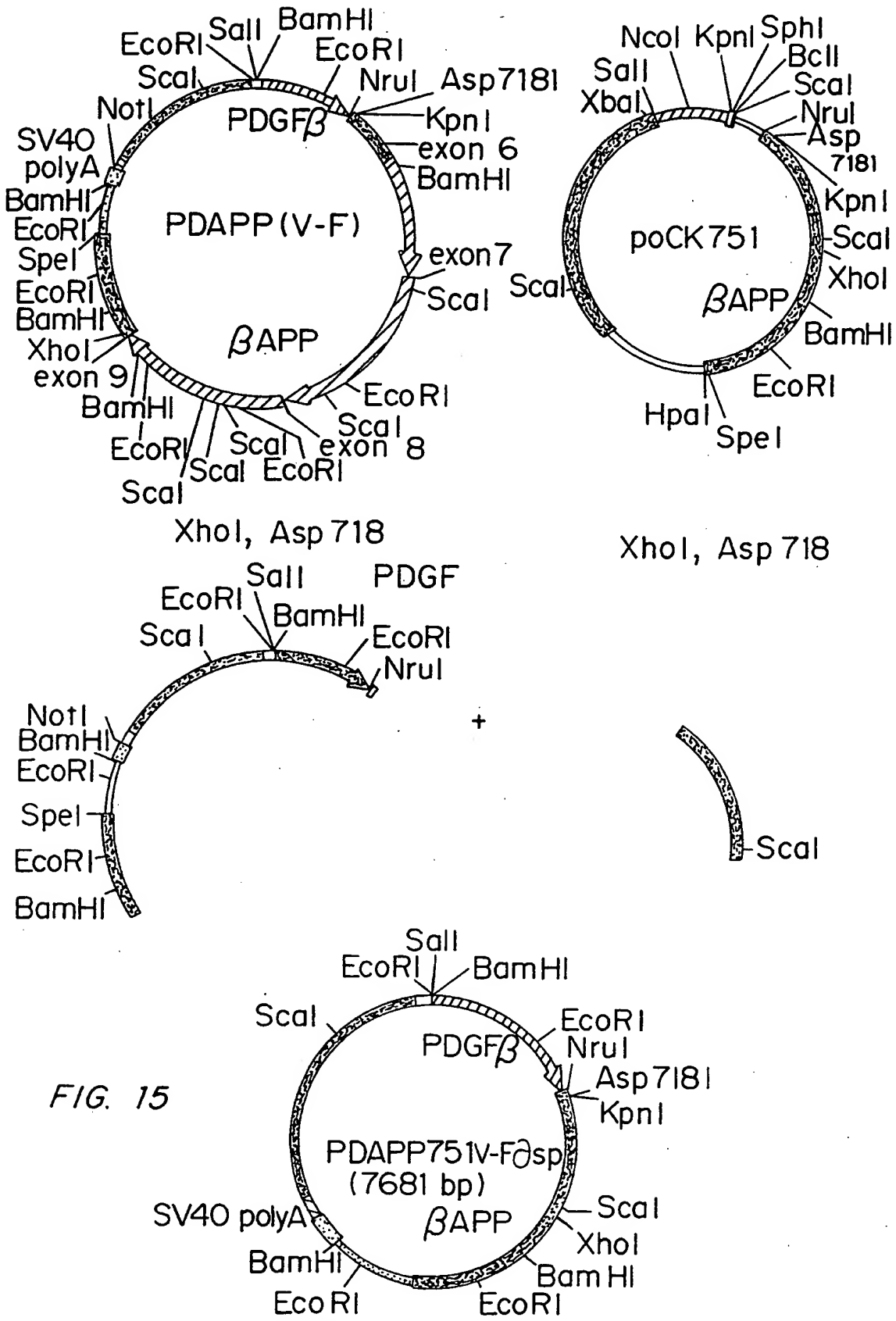

EcoRI
SalI
BamHI
EcoRI
ScaI
NruI
Asp7181
NotI
PDGFβ
KpnI
SV40
polyA
exon 6
BamHI
BamHI
EcoRI
PDAPP(V-F)
SpeI
exon7
EcoRI
ScaI
BamHI
βAPP
XhoI
exon 9
EcoRI
BamHI
ScaI
EcoRI
ScaI
exon 8
ScaI
ScaI
EcoRI
XhoI, Asp 718
NcoI
KpnI
SphI
BclI
SalI
XbaI
ScaI
NruI
Asp
7181
KpnI
poCK751
ScaI
ScaI
XhoI
βAPP
BamHI
EcoRI
HpaI
SpeI
XhoI, Asp 718
SalI
PDGF
EcoRI
BamHI
ScaI
EcoRI
NruI
NotI
BamHI
EcoRI
SpeI
EcoRI
BamHI
+
ScaI
SalI
EcoRI
BamHI
ScaI
EcoRI
PDGFβ
NruI
Asp7181
KpnI
PDAPP751V-FΔsp
(7681 bp)
SV40 polyA
βAPP
ScaI
XhoI
BamHI
Bam HI
EcoRI
EcoRI

FIG. 15

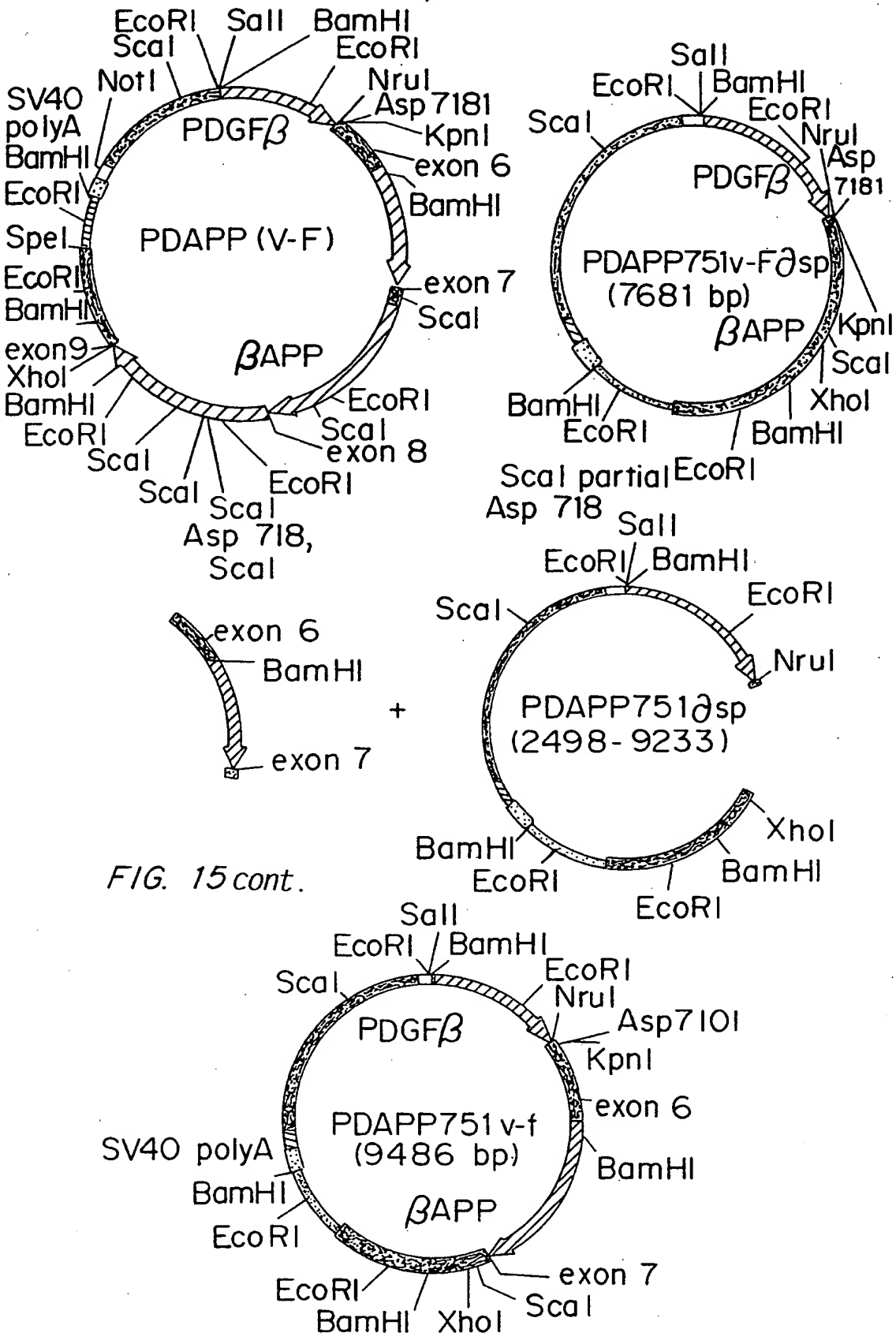

EcoRI
SalI
BamHI
ScaI
EcoRI
NotI
NruI
SV40
polyA
PDGFβ
Asp 7181
BamHI
KpnI
EcoRI
exon 6
PDAPP (V-F)
BamHI
SpeI
EcoRI
exon 7
BamHI
ScaI
exon9
βAPP
XhoI
BamHI
EcoRI
ScaI
EcoRI
ScaI
exon 8
ScaI
ScaI
EcoRI
Asp 718,
ScaI
SalI
EcoRI
BamHI
ScaI
PDGFβ
EcoRI
NruI
Asp
7181
PDAPP751v-Fδsp
(7681 bp)
βAPP
KpnI
ScaI
XhoI
BamHI
EcoRI
BamHI
ScaI partial
EcoRI
Asp 718
exon 6
BamHI
exon 7
+
SalI
EcoRI
BamHI
ScaI
EcoRI
NruI
PDAPP751δsp
(2498-9233)
XhoI
BamHI
EcoRI
BamHI
EcoRI
SalI
EcoRI
BamHI
ScaI
EcoRI
NruI
PDGFβ
Asp7101
KpnI
exon 6
PDAPP751v-f
(9486 bp)
SV40 polyA
BamHI
βAPP
EcoRI
EcoRI
exon 7
BamHI
XhoI
ScaI

FIG. 15 cont.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09857

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 C12N15/12 C12N15/62 C07K14/47 A01K67/027
C12Q1/68 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 373, 9 February 1995, MACMILLAN JOURNALS LTD., LONDON,UK, pages 523-527, XP002013196 D. GAMES ET AL.: "Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein" see page 527, left-hand column, line 16 - line 21 --- -/--	1-7, 21-23, 26,27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
13 September 1996	20.09.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE NEW ENGLAND JOURNAL OF MEDCINE, vol. 332, no. 22, 1 June 1995, MA. MED. SOC., BOSTON, MA,US, pages 1512-1513, XP000602018 R.E. TANZI ET AL.: "Clinical implication of basic research - A promising animal model of Alzheimer disease" see page 1512, left-hand column, line 1 - right-hand column, line 5 ---	1-27
Y	NEUROBIOLOGY OF AGING, vol. 16, no. 4, 1995, PERGAMON, ELSEVIER SCIENCE INC., NEW YORK, US, pages 685-699, XP002013197 D.S. HOWLAND ET AL.: "Mutant and native human beta-amyloid precursor proteins in transgenic mouse brain" see the whole document ---	24,25
Y	WO,A,94 23049 (UNIV JOHNS HOPKINS) 13 October 1994 see the whole document ---	24,25
Y	WO,A,95 11968 (ATHENA NEUROSCIENCES, INC., ELLI LILLY AND COMPANY) 4 May 1995 see the whole document ---	24,25
Y	WO,A,95 14769 (UNIVERSITY OF SOUTH CALIFORNIA) 1 June 1995 see the whole document ---	24,25
A	WO,A,93 14200 (TSI CORP) 22 July 1993 see the whole document ---	1-27
A	NEURON, vol. 14, March 1995, CELL PRESS,CAMBRIDGE, MA,USA, pages 661-670, XP000602022 M. CITRON ET AL.: "Generation of amyloid beta protein from its precursor is sequence specific" cited in the application see the whole document ---	1-27
P,A	J. BIOL. CHEM., vol. 270, no. 47, 24 November 1995, AM. SOC. BIOCHEM. MOL.BIOL.,INC.,BALTIMORE,US, pages 28257-28267, XP002013198 E.M. ROCKENSTEIN ET AL.: "Levels and alternative splicing of amyloid beta protein precursor (APP) transcripts in brains of APP transgenic mice and humans with Alzheimer's disease" cited in the application see the whole document --- -/--	1-27

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	NEUROBIOLOGY OF AGING, vol. 17, no. 2, - 1996 PERGAMON, ELSEVIER SCIENCE INC., NEW YORK, US, pages 153-171, XP000602061 B.D. GREENBERG ET AL.: "APP transgenesis: Approaches toward the development of animal models for Alzheimer disease neuropathology" see page 161, right-hand column, line 10 - line 63 -----	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 96/09857

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